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ABSTRACT

Establishment and maintenance of tissue polarity is a fundamental property of epithelial cells and loss of this property is a hallmark of the breast cancer phenotype. We have used a 3-dimensional culture assay in which malignant human breast cells can be phenotypically reverted to a non-malignant phenotype when PI3 kinase (PI3K) activity is inhibited. We show here that Akt and Rac1 act as downstream effectors of PI3K and function as control points of cellular proliferation and tissue polarity, respectively. Signaling through these two effectors is sufficient to prevent restoration of a normal phenotype. We reveal key events downstream of PI3K that act synergistically to maintain tissue polarity and when disrupted result in a malignant phenotype. We also find that activation of Akt1 can prevent invasive behavior in vitro. Activated Akt1 in human breast cancer cells affects cell motility and invasiveness by targeting the tumor suppressor tuberous sclerosis complex 2 (TSC2) for degradation, leading to reduced Rho-GTPase activity. TSC2 thus acts as a downstream target of Akt1 to regulate breast cancer cell motility and invasion, suggesting the need for caution in designing therapies targeting the function of individual genes in epithelial tissues.

INTRODUCTION

Tumor progression is a process involving the accumulation of multiple genetic lesions and consequently the malfunctioning of intracellular and extracellular regulatory machineries. The aberrant alterations cause uncontrollable cell propagation, loss of tissue-specific differentiation, and escape from monitoring mechanisms. Loss of proliferation control has been shown to be the driving force of cell transformation and tumor development. It is well established that loss of polarity and tissue disorganization are hallmarks of epithelial cancers. Mammary epithelial cells lose polarity as they progress toward malignancy. Epithelial polarization is affected by extracellular matrix and surrounding microenvironment, and abnormality in this aspect as found in many epithelial cancers has profound effects. Studies have demonstrated that defects in polarity are linked to control of cell proliferation and some characteristics of malignancy. PI3 kinase (PI3K) has been shown to be able to transform normal cells in vitro, and has been found to be overexpressed in many cancers including that of breast. However, the known complexity of PI3K signaling pathways implies that downstream portions of these pathways could be functionally distinguished. By using a 3-dimensional laminin-rich extracellular matrix (3D lrECM) culture assay and a human mammary epithelial cell progression series, we previously showed that polarity could be restored and proliferation reduced in human mammary epithelial cells when PI3K overactivation was diminished. In order to further dissect the molecular mechanisms of PI3K-mediated reversion and to distinguish if proliferation and polarity are two separable events downstream of PI3K signaling pathways that eventually integrate and synergistically contribute to tumor development, the activities of effectors which regulate proliferation and polarity, such as Akt and Rac1, were genetically manipulated and their effects on reversion phenotypes tested in the 3D lrECM assay.

Metastasis is one of the major characteristics of malignancy, the process by which cancer cells undergo an aberrant and complex process to become invasive and ultimately acquire the ability to metastasize to distant organs. The evolutionarily conserved serine/threonine kinase Akt is one of the major downstream effectors of PI3K and one of the most frequently activated protein kinases in human cancers. Hyperactivation of Akt is associated with resistance to apoptosis and increase in cell proliferation and cellular energy metabolism via substrate phosphorylation. The role of Akt in PI3K-mediated regulation of cell proliferation and survival as well as promotion of tumor growth in animal models is well established. Activation of Akt is also correlated with an alteration in cell migration and invasion in several mammalian systems.

While characterizing other malignant phenotypes induced by expressing constitutively active Akt in the human mammary epithelial cell line HMT-3522 T4-2, we found that up-modulation of Akt activity had profound effects on cell motility, invasion, and actin cytoskeleton in addition to the greatly increased cell proliferation and survival observed in both in vitro and in vivo assays. We then further characterized the downstream pathways downstream of an up-modulated Akt, which will provide novel insights into the molecular mechanisms of its role in tumorigenesis when dysregulated.

BODY

Polarity and proliferation are controlled separately by distinct pathways downstream of PI3K.

We previously reported that PI3K activity is up-regulated in HMT-3522 T4-2 (T4-2) cells as compared to their non-malignant counterpart, HMT-3522 S1 (S1), and that down-modulation of PI3K signaling by the use of specific PI3K inhibitors LY294002 or wortmannin is sufficient to reduce both cellular proliferation and anchorage-independent growth in T4-2 cells. Interestingly, T4-2 cells reverted by inhibition of PI3K in 3D lrECM also have restored apicobasal polarity and exhibit basolateral localization of both PI3K and its phospholipid product PIP3. The fact that PI3K inhibition affected a number of distinct signaling pathways demonstrates that signal transduction pathways downstream of PI3K may be integrated when cells are grown in the physiological 3D lrECM context. Consistent with this model, we found that reversion of T4-2 cells by PI3K inhibition was also associated with down-modulation of EGFR and β1 integrin (Figure 1A) and increased expression of PTEN, the PI3K antagonist, the latter occurring only in the presence of 3D lrECM (Figure 1B). These observations suggest that dysregulation of PI3K has profound effects on both proliferation and polarity.

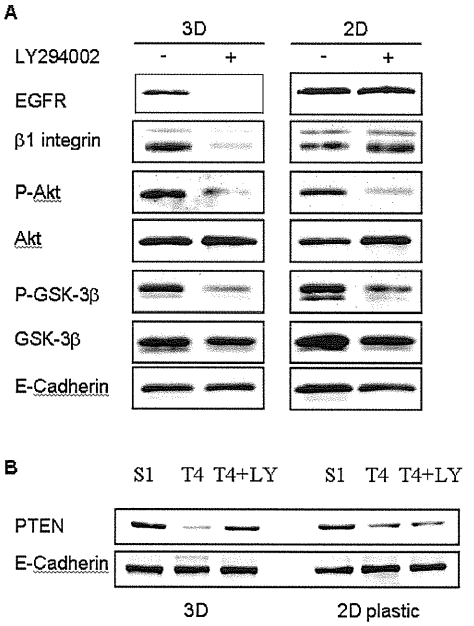

Figure 1. Inhibition of PI3K signaling results in cross-modulation of other signaling pathways and intermediates. Cell lysates from S1, T4-2, and T4-2 cells + LY294002, grown either in 3D lrECM or on 2D tissue culture plastic for 10 days, were subjected to western blotting against the indicated proteins; E-cadherin was used as loading control.

As a follow-up to these results, we attempted to determine if T4-2 cells could be also reverted by over-expression of PTEN, a phospholipid phosphatase and antagonist of PI3K signaling. However, cells died upon overexpression of the PTEN-GFP construct and a stable population could not be obtained.

The primary consequence of PI3K activation is the generation of PIP3 in the membrane, which functions as a second messenger to activate downstream pathways which involve Akt and other proteins such as SGK, RSK, atypical PKC [1], and Rac1. Akt has been shown to be one of the most important effectors of PI3K effects on cellular proliferation and apoptosis [2]. PI3K can also affect Rac1 activity by regulating the activity of its GTP/GDP exchange factors (GEF) such as Tiam1 and Vav1 [3]. Rac1 is one of the major components of an evolutionarily conserved protein complex (PAR3/PAR3/aPKC) that plays an important role in tight junction formation and regulation of the development of

polarity [4]. When Rac1 is dysregulated, polarity is lost due to a failure to asymmetrically deposit the ECM component laminin [5]. An alternative splice variant of Rac1, Rac1b, which has an increased GTP/GDP exchange rate, is also highly expressed in breast and colon carcinoma [6]. As effectors downstream of PI3K could regulate both proliferation and perhaps cell polarity in addition, we hypothesized that the signaling pathways of PI3K might bifurcate into functionally discrete signaling branches, one controlling proliferation, and another controlling polarity.

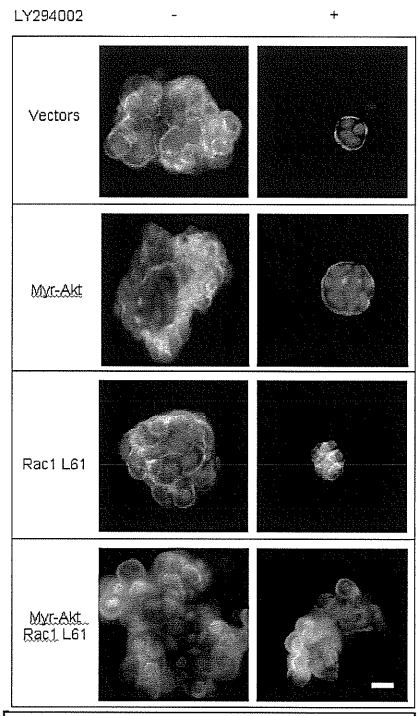

Figure 2. Effects of constitutive activation of Akt and Rac1 on LY294002-mediated reversion. The transfectants described above were cultured in 3D lrECM for 10 days. Basal polarity was assessed by immunofluorescence against α6 integrin. Scale bar, 10 μm.

In order to address this hypothesis, we established stable populations of T4-2 cells overexpressing: 1) constitutively active myrisolated Akt (myr-Akt); 2) constitutively active Rac1 L61; 3) dominant negative Rac1 N17; and 4) both myr-Akt and Rac1 L61. We additionally attempted to generate stable cell lines of T4-2 cells expressing adenoviral constructs of Rac1 V12 and N17 but were unsuccessful. We then determined the effects of these constructs on the reversion phenotypes mediated by the PI3K-specific inhibitor LY294002 in the 3D lrECM culture assay (Figure 2). We observed that constitutive activation of Akt rendered T4-2 cells more resistant to the reduction of proliferation induced by PI3K inhibition-mediated reversion. Intriguingly, constitutively active Akt did not interfere with the re-establishment of basal polarity of the revertant colonies although it significantly increased these cells' ability to grow in an anchorage independence assay (Figure 3).

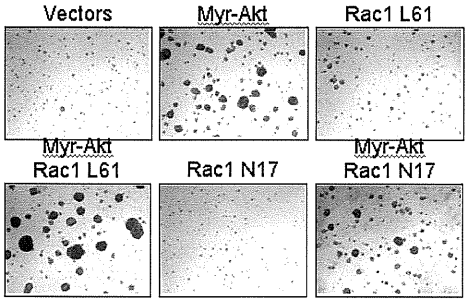

Figure 3. Constitutive activation of Akt but not Rac1 promotes anchorage-independent growth; activation of both has a synergistic effect. T4-2 cells transfected as described were cultured in methylcellulose for 3 weeks.

To further investigate if PI3K/Akt mediated effects on proliferation may be separated from their effects on polarity, we interrogated mTor (a mammalian target of rapamycin), a major downstream effector of Akt and a regulator of cell proliferation and growth [7]. We treated T4-2 cells with rapamycin at a concentration sufficient to abrogate the activity of mTor and found that this treatment significantly reduced cellular proliferation to a level similar to that of T4-2 cells reverted by PI3K inhibition but could not restore basal polarity (data not shown).

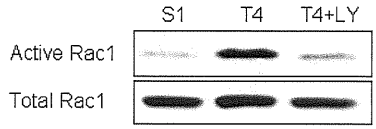

Figure 4. Rac1 activity is increased in T4-2 as compared to S1; PI3K inhibition reduces Rac1 activity. Rac1 activity was analyzed by recombinant PAK-GST-CD fusion protein pulldown from lysates of 10-day 3D lrECM cultures of S1, T4-2 treated with vehicle, and T4-2 treated with LY294002.

The above results are consistent with another recent study investigating the effects of overexpression of activated Akt in MCF-10A, a transformed, non-malignant mammary epithelial cell line. This study showed that activation of the Akt pathway increased the size and cell number of the cysts formed but that basal polarity was still maintained in 3D lrECM culture [8]. All of these observations provide strong evidence suggesting that the regulation of proliferation through Akt is not tied to regulation of polarity control and that signaling pathways regulating proliferation and polarity originating at PI3K might branch off at the level of Akt, implying that another pathway(s) parallel to Akt but downstream of PI3K might exist to control polarity.

We then sought to investigate candidates for the effector downstream of PI3K which might control polarity. We found that levels of activated Rac1 decreased upon down-modulation of PI3K activity (Figure 4). We also found that, in contrast to cells expressing constitutively active Akt, normalization of PI3K activity could substantially reduce cell proliferation to the level of treated vector control or parental cells. However, basal polarity was completely lost if the downregulation of Rac1 activity was prevented by the expression of constitutively active Rac1 (Figure 2). Furthermore, when active Akt and Rac1 were coexpressed, PI3K inhibition no longer reverted; cells continued to proliferate and lacked basal polarity (Figure 2). These observations have three significant implications. First, when PI3K signaling is dysregulated, Rac1 acts downstream to mediate the loss of tissue polarity in a pathway separate from that which controls cell proliferation; second, aberrant proliferation (through increased Akt activity) and loss of polarity (via up-regulated Rac1 activity) constitute the minimal signaling inputs required through PI3K to cause tumor phenotypes; and third, disruption of polarity is one of the major requirements or enhancers for epithelial tumorigenesis once cell proliferation becomes uncontrolled.

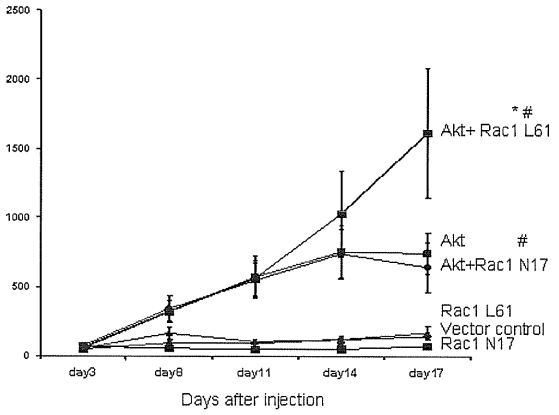

Figure 5. Myr-Akt and Rac1 L61 double transfectants synergistically promote tumor formation in nude mice. T4-2 transfectants were injected subcutaneously into the rear flanks of nude mice and tumor volume was measured twice a week. *, Akt+Rac1 L61 vs Akt or Akt+Rac1 N17 ($p<0.02$); #, Akt alone, Akt+Rac1 L61, or Akt+Rac1 N17 vs. Rac1 L61, Rac1 N17, or vector control ($p<0.01$).

To further test this model, we injected the above described T4-2 transfectants (vector control; myr-Akt; Rac1 L61; Rac1 N17; myr-Akt and Rac1 L61; and myr-Akt and Rac1 N17) into nude mice (n=6 for each transfectant) and observed the tumor growth *in vivo*. We found that

transfection with constitutively active Akt but not Rac1 L61 resulted in a significant increase in tumor size and that double transfection with constitutively active Akt and Rac1 L61 acted synergistically to lead to even larger tumors than active Akt alone (Figure 5).

Our results suggest a model for a mechanism of PI3K-induced tumorigenesis in mammary epithelial cancers. PI3K acts as one of the signaling nodes to be activated by and relay the signals from overactivated cell surface receptor such as receptor/protein tyrosine kinases and integrins. The signals from PI3K then bifurcate into function-specific and possibly mutually independent pathways controlling proliferation and polarity. Activation of cell cycle-promoting or anti-apoptosis pathway(s) through Akt or SGK increase cell proliferation, while activation of polarity pathway(s) through Rac1 causes disruption of tissue polarity or architecture. The convergence of these two phenotypic effects induced by these different pathways synergistically leads to tumor development. Whether or not this model could be generalized to other oncogenes or kinases with multiple functions remains to be tested.

Mechanism of Akt inhibition of breast cancer cell invasion

We assessed whether deregulation of Akt signaling could affect cell motility and invasion in addition to cell proliferation and survival. T4-2 cells transfected with empty vector or myr-Akt were subjected to anchorage-independence assays in soft agar or methyl cellulose. As expected, cells overexpressing myr-Akt had significantly increased colony number and size (Figures 6A and B). Myr-Akt cells cultured in 3D lrECM also had larger colony sizes as previously shown [9]. The increases in cell survival and growth are due to an increase in cell proliferation and a decrease in apoptosis as assessed by Ki67 and TUNEL immunostaining, respectively (data not shown). As previously described, activated Akt also resulted in a considerable increase in average tumor volume as well as an increase in growth rate as compared to the vector control ($p<0.001$, Figure 6C). These results all suggest that the Akt signaling pathway is a driving force for cell survival and tumor growth.

To determine if Akt dysregulation could also affect cell invasiveness, T4-2 cells expressing myr-Akt and vector control were subjected to an invasion assay. Unexpectedly, we found that constitutive activation of Akt reduced the invasion of T4-2 cells through Matrigel-coated Boyden chambers (Figure 6D, n=8).

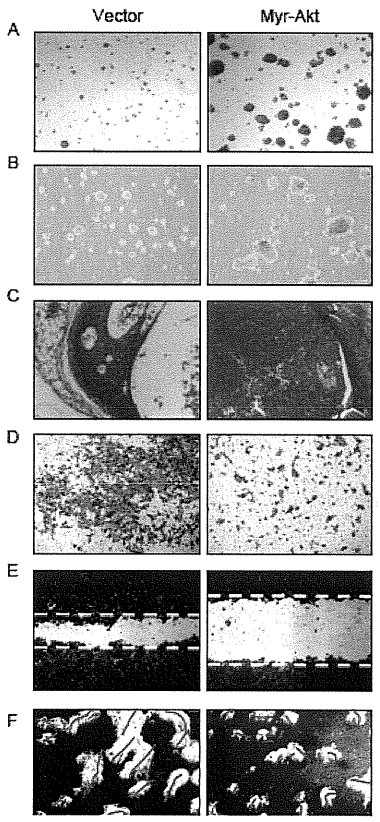

Figure 6. Upregulation of Akt activity enhances cell survival and tumor formation but inhibits cell invasiveness and motility.

Since altered cell motility is a primary driving force of invasiveness, wound-healing and cell migration assays were employed to directly assess the effect of the constitutive activation of Akt on motility. We found that myr-Akt significantly inhibited cell motility in the wound healing assay (Figure 6E). To observe the compromised motility in real time, we monitored the tracks of cell random migration for 24 hours (Figure 6F). We found that Myr-Akt cells migrated much less than control cells in concordance with our previous results. These results suggest that hyperactivation of Akt signaling negatively regulates cell migration and invasion.

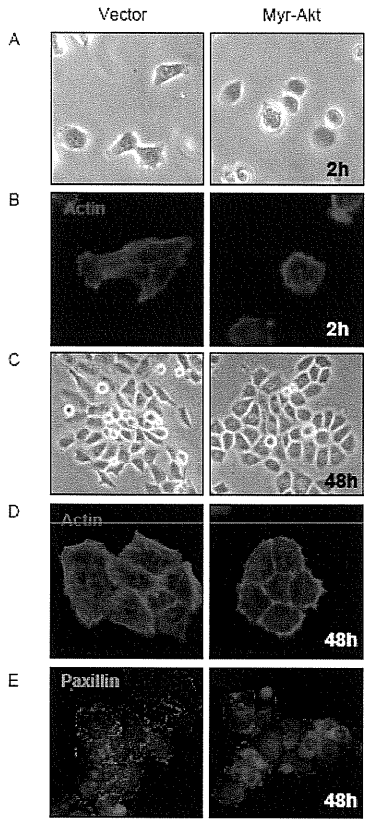

Figure 7. Cell spreading, actin cytoskeleton, and focal adhesions are drastically altered by overexpression of constitutively active Akt

To confirm the role of Akt in the reduction of cell invasion and motility we observed, we asked if the catalytic activity of Akt was required to observe this phenotype. A dominant negative Akt construct mutated at serine 473 and threonine 308 was generated and transduced into T4-2 cells. Transduction of dominant negative Akt did not have a significant effect on either cell invasion or motility, even though its overexpression remarkably reduced the phosphorylation of its downstream targets and caused smaller colonies in 3D lrECM assay, suggesting the kinase activity of Akt is necessary for cell motility and invasion inhibition (data not shown).

Cell extensions are a prerequisite for the onset and maintenance of cell motility in normal and cancer cells [10]. We found that cell adhesion to culture plates and spreading was significantly delayed when active Akt was overexpressed (Figure 7A). These cells formed few extensions throughout 2D culture and eventually formed compact colonies with smoother edges as compared to control (Figure 7C), suggesting that cell spreading was fundamentally changed.

Cell movement is a dynamic, orchestrated, cyclical process in which moving cells undergo drastic changes in cell shape, and involves the creation of actin-rich protrusions, formation and disassembly of adhesive complexes, and establishment of migration polarity [10, 11]. Malfunctions occurring in any of those steps can greatly affect cell motility. As actin polymerization is one of the major driving forces of cellular movement [12], we examined actin patterns and their dynamics in the vector control and myr-Akt T4-2 cells. In contrast to vector control cells, cells overexpressing active Akt had increased cortical actin staining but exhibited a stark reduction in actin stress fibers at both 48 hours and early time points after plating (Figures 7B, D). When scratch wounds were made on a confluent monolayer to re-initiate cell movement and the healing

process, there were much fewer actin-rich projections towards the wound in the myr-Akt cells as compared to the vector control cells.

Since focal adhesion formation is required for cell attachment and serve as points of contact for cell spreading and of traction over which the body of the cell moves [13], we examined focal adhesion formation by immunostaining against the focal adhesion component, paxillin. We discovered that overactivation of Akt significantly decreased the formation of focal adhesions without any effect on endogenous paxillin expression (Figure 7E). These results suggest that overactive Akt signaling interferes with the process of focal adhesion formation, a defect that might be relevant to the previously observed phenotype of decrease of cell invasiveness and mobility.

Since the assembly and organization of the actin cytoskeleton are controlled by Rho GTPases, and the formation of stress fibers and focal adhesions are hallmarks of Rho activation [14], we assessed Rho activity using a Rhotekin-GST fusion protein pulldown assay. Consistent with the observed phenotype of stress fiber loss, levels of active Rho were significantly lower in myr-Akt cells, beginning from 2 hours after cell plating on tissue culture plastic (Figure 8). To determine if the change in actin cytoskeleton phenotype correlates with Rho activity, cells plated on coverslips were stained with phalloidin at the same time points as pulldown assays. At 2 hours, vector control, but not myr-Akt cells, spread well and had already formed stress fibers (Figure 7B). There was no significant difference in Rac1 and Cdc42 activities in parallel lysates, even though Akt was found to phosphorylate and inhibit Rac1 activity [15]. These results suggested that overactivated Akt specifically inhibits Rho activity and that decreased Rho activity might play a role in constitutively active Akt-mediated defects in motility and invasiveness. However, cell spreading, focal adhesion formation, and actin cytoskeleton phenotype of dominant-negative Akt-transfected T4-2 cells were very similar to vector control T4-2 cells. These results suggest that increased catalytic activity of Akt signaling profoundly affects Rho activity and actin cytoskeleton, and consequently, cell motility and invasiveness.

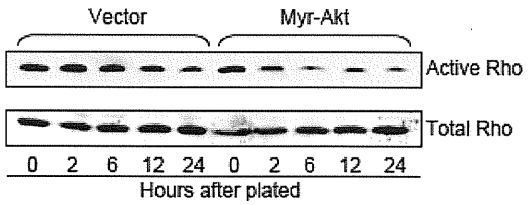

Figure 8. Constitutive activation of Akt results in a decrease in Rho activity.

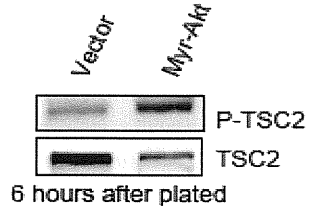

Figure 9. Constitutive activation of Akt results in an increase in phosphorylated TSC2 and a decrease in total TSC2.

TSC1 and TSC2 are tumor suppressors and TSC2 is a substrate of Akt. Both proteins are regulated by binding to protein 14-3-3 as a consequence of Akt-mediated phosphorylation [16]. Since TSC2 or TSC1 has been shown to activate Rho activity and induce active Rho phenotypes [17, 18], we postulated that Akt overactivation might lead to dysregulation of TSC2 or TSC1, which in turn could reduce Rho activation. To test this possibility, we examined the levels of total and phosphorylated TSC1 and TSC2 in T4-2 cells expressing myr-Akt or vector control and found that

TSC2 was highly phosphorylated at threonine 1462 in myr-Akt cells (Figure 9). We also found that the total expression level of TSC2 was significantly decreased in myr-Akt cells but not dominant negative Akt cells (Figure 9 and data not shown). TSC2 levels are negatively correlated with levels of myr-Akt expression and Rho activity as a function of time. At the same time, we did not observe any significant differences in TSC1 expression. These results suggest that activation of Akt signaling causes phosphorylation of TSC2 with concomitant down-modulation of TSC2 expression, implying that low levels of TSC2 might result in a decrease in Rho activation.

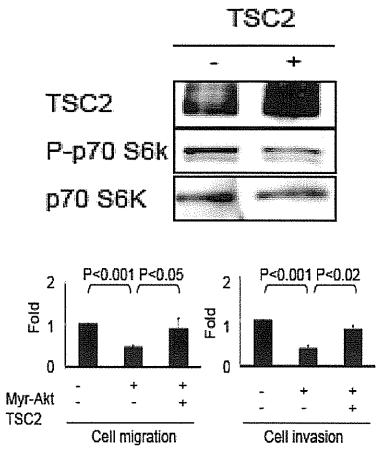

Figure 10. TSC2 acts downstream of Akt to regulate cell invasiveness and motility.

To determine if down-modulated TSC2 might play a role in the defects in cell motility and invasion observed in myr-Akt cells, wild-type TSC2 was stably expressed in vector control and Myr-Akt cells (Figure 10). Overexpression of TSC2 in vector control cells resulted in a significant decrease in the phosphorylation of p70S6K (Figure 10) and decreased colony size when cells were grown in 3D lrECM, consistent with published results indicating that TSC2 is upstream of mTor and p70S6K and negatively regulates cell proliferation and growth [19-23]. Overexpressing TSC2 in myr-Akt cells rescued the defective cell motility and invasiveness phenotype (Figure 10). These results indicate that although TSC2 acts as a tumor suppressor that inhibits cell growth and proliferation, it is also required for cell motility and invasion.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that constitutive activation of Rac1 signaling prevents the restoration of polarity in the phenotypic reversion of T4-2 cells mediated by PI3K inhibition in 3D lrECM without significant effect on cell proliferation.

- Determined that constitutive activation of Akt partially prevents the reduction of cell proliferation in the phenotypic reversion of T4-2 cells mediated by PI3K inhibition in 3D lrECM without any effects on polarity.

- Determined that constitutive activation of both Rac1 and Akt is sufficient to prevent the phenotypic reversion of T4-2 cells mediated by PI3K inhibition in 3D lrECM as assessed by lack of acquisition of cell polarity and failure to growth arrest.

- Determined that constitutive activation of Akt increases ability to grow anchorage-independently and that constitutive activation of both Akt and Rac1 synergistically promotes growth in the same assay.

- Determined the roles of constitutively active and dominant negative Akt in the regulation of cell motility and invasion.
- Determined the effects of constitutively active and dominant negative Akt on cell spreading, actin cytoskeleton, and focal adhesion formation.
- Determined that Rho activity is altered when constitutively active Akt is overexpressed.
- Determined that modulation of Akt signaling affects the stability of TSC2.
- Determined that TSC2 acts as a downstream target of Akt to control cell motility and invasion.
- Accomplished *in vivo* experiments in mice to confirm *in vitro* observations.

REPORTABLE OUTCOMES

- Stable populations of cells expressing: myr-Akt; Rac1 L61; Rac1 N17; and myr-Akt and Rac1 mutants were established.
- Posters and abstracts:
    - Liu H, Radisky DC, Wang F, Bissell MJ. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. Era of Hope Meeting Breast Cancer Research Program of the Department of Defense, June 2005, Philadelphia, Pennsylvania.
    - Liu H, Radisky DC, Bissell MJ. Activation of Akt inhibits cell invasiveness and motility and alters actin and microtubule cytoskeletal networks in mammary epithelial cells. Gordon Research Conference of Signaling by Adhesion Receptors, June 2004. Bristol, RI.
    - Liu H, Radisky DC, Wang F, Bissell MJ. Polarity and Proliferation are Controlled Independently by Rac1 and Akt Signaling Pathways in Malignant and Reverted Breast Epithelial Cells in Three Dimensions. Annual Meeting of American Society of Cell Biology, December 2003. San Francisco, CA.
- Publications:
    - Liu H, Radisky DC, Wang F, Bissell MJ. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. J Cell Biol. 2004 Feb 16; 164(4): 603-12.

- Liu H, Radisky DC, Nelson C, Zhang H, Fata JE, Roth R, Bissell MJ. Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2. PNAS 2006 March 14: 103 (11): 4134-4139.

- Training:

  - Hong Liu received a PhD from UC Berkeley in June 2005.

  - Hong Liu is currently employed as a postdoctoral fellow in the lab of Nobel Laureate Dr. Michael Bishop at UCSF.

CONCLUSIONS

We have used the 3D lrECM cell culture assay and genetic manipulation of Akt and Rac1 activity to investigate the role of PI3K signaling in tumorigenesis, signaling reorganization, and polarity in mammary epithelial cells. Our elucidation that polarity and proliferation are controlled independently by separable events downstream of PI3K sheds light on the molecular mechanisms by which increased proliferation and loss of tissue polarity may act discretely and affect various cell phenotypes even under one overactive kinase signaling pathway. The aberration of both phenotypes is required to produce the malignant phenotype, which might be generalized to other signaling pathways or models to dissect the process of tumor development.

Our results also demonstrate that the activation of the Akt signaling pathway has a profound effect on cell invasiveness and motility. The inhibition of invasiveness as a result of constitutive activation of the Akt pathway might be due to the negative regulation of cell motility as mediated by TSC2 and Rho signaling pathways. The elucidation of novel targets in these pathways may shed light on the molecular mechanisms of invasion and the role of Akt in their regulation.

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APPENDICES

1. Liu H, Radisky DC, Wang F, Bissell MJ. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. J Cell Biol. 2004 Feb 16; 164(4): 603-12.

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Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells

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Loss of tissue polarity and increased proliferation are the characteristic alterations of the breast tumor phenotype. To investigate these processes, we used a three-dimensional (3D) culture system in which malignant human breast cells can be reverted to a normal phenotype by exposure to inhibitors of phosphatidylinositol 3-kinase (PI3K). Using this assay, we find that Akt and Rac1 act as downstream effectors of PI3K and function as control points of cellular proliferation and tissue polarity, respectively. Our results also demonstrate that the PI3K signaling pathway is an integral component of the overall signaling network induced by growth in 3D, as reversion affected by inhibition of PI3K signaling also down-modulates the endogenous levels of β1 integrin and epidermal growth factor receptor, the upstream modulators of PI3K, and up-regulates PTEN, the antagonist of PI3K. These findings reveal key events of the PI3K pathway that play distinct roles to maintain tissue polarity and that when disrupted are instrumental in the malignant phenotype.

Introduction

Class I phosphatidylinositol 3-kinase (PI3K) is activated by growth factor–responsive tyrosine kinases such as epidermal growth factor receptor (EGFR; Grant et al., 2002) and integrin-responsive kinases such as focal adhesion kinase (FAK; Chen and Guan, 1994). Activated PI3K leads to the production of membrane-associated phosphatidylinositol 3,4,5-trisphosphate (PIP3), which in turn causes the recruitment to the cell membrane and subsequent activation of a number of signaling molecules (Vivanco and Sawyers, 2002). PI3K is a key mediator in processes that regulate cell orientation; for both *Dictyostelium* and cultured human leukocytes, directionality of chemotaxis is controlled by polarization of PIP3 to the leading edge of the cell (Servant et al., 2000; Funamoto et al., 2002; Wang et al., 2002b). PI3K has been found to be constitutively up-regulated in a substantial fraction of human breast cancers (Vivanco and Sawyers, 2002), and overexpression of PI3K in cultured nonmalignant human mammary epithelial cells is sufficient to confer a malignant phenotype (Zhao et al., 2003).

During tumor progression, tissue polarity is lost and control of proliferation is compromised (Fish and Molitoris, 1994; Reichmann, 1994; Bissell and Radisky, 2001), and although these two phenomena have been suggested to be linked, previous investigations have not revealed the extent to which the increased cellular proliferation in tumors can directly produce tissue disorganization, and to what extent loss of polarity is an independent function of deregulated signaling pathways downstream of the oncogenic signal(s). To dissect the molecular mediators of these processes we have used an assay (Petersen et al., 1992) in which human mammary epithelial cells from the HMT-3522 tumor progression series are cultured in a physiologically relevant, three-dimensional (3D) laminin-rich basement membrane (lrBM). When cultured in 3D lrBM, the phenotypically normal, nonmalignant HMT-3522 S-1 (S-1) cells undergo growth arrest, produce an endogenous basement membrane, and form polarized acinus-like structures, very similar to primary cells from reduction mammoplasty. In contrast, the

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Key words: three-dimensional cultures; Akt; Rac1; tumor reversion; tissue polarity

Abbreviations used in this paper: 2D, two dimensional; 3D, three dimensional; EGFR, epidermal growth factor receptor; GSK 3β, glycogen synthase kinase-3β; lrBM, laminin-rich basement membrane; Myr-Akt, myristoylated Akt (constitutively active Akt); PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate.

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malignant HMT-3522 T4-2 (T4-2) cells continue to proliferate into apolar, amorphous structures, similar to structures formed by primary tumor cells in this assay (Petersen et al., 1992). In comparison to S-1 cells, expression levels of EGFR and β1 integrin in T4-2 cells are greatly increased, and down-regulation of these signaling pathways in T4-2 cells grown in 3D lrBM can restore the formation of polarized acinus-like structures, resulting in a reversion similar to the normal phenotype of the S-1 cells (Weaver et al., 1997; Wang et al., 1998).

As PI3K is activated downstream of both EGFR and β1 integrin (Chen and Guan, 1994; Lee and Juliano, 2000; Grant et al., 2002), we hypothesized that the phenotypic reversion affected by down-modulation of EGFR/β1 integrin signaling in T4-2 cells was due to attenuation of PI3K activity. We showed previously that even highly malignant metastatic cancer cells, cultured in 3D lrBM, could be reverted to a normal phenotype by inhibition of PI3K, if treatment with PI3K inhibitors was performed in combination with appropriate manipulation of other signaling pathways (Wang et al., 2002a). Here we use inhibition of PI3K alone to dissect the signaling pathways that control proliferation and polarity in breast tumor cells. Our results reveal a new functional link between extracellular signaling mediators and tissue function that provides insight into processes that control the malignant phenotype if imbalanced. We also show that the PI3K and its lipid product, PIP3, are relocalized to the basal surface of the acini when the malignant cells are reverted in lrBM, a process that may play a role in integration of signaling pathways in reformation of polarity.

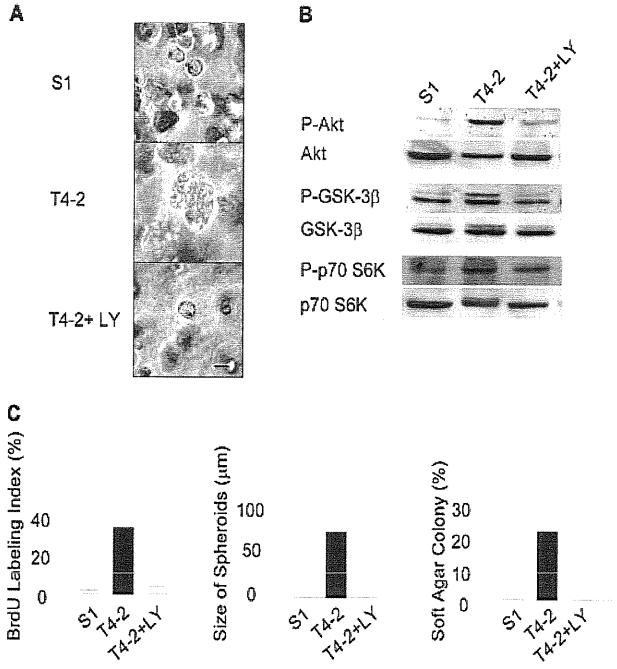

Figure 1. **Attenuation of PI3K activity results in phenotypic reversion of HMT-3522 T4-2 human mammary tumor cells cultured in 3D BM.** (A) Phase contrast micrographs of 10-d 3D lrBM cultures of phenotypically normal (S-1), malignant (T4-2), and T4-2 cells treated with 8 μM PI3K inhibitor, LY294002 (T4-2+LY). Bar, 20 μm. (B) Cell lysates from 10-d 3D lrBM cultures were analyzed for phosphorylated Akt (serine 473)/total, phosphorylated GSK-3β (serine 9)/total, and phosphorylated p70 S6 kinase (threonine 389)/total by Western blot. (C) Inhibition of PI3K causes a reduction in cellular proliferation (left, BrdU labeling assay, $n = 3$), colony size (center, 50 colonies assessed for each experiment, $n = 3$), and anchorage-independent growth (right, soft agar assay, colonies scored positive when >50 μm, $n = 3$).

Results

Down-modulation of PI3K activity results in phenotypic reversion of human mammary tumor cells

We found previously that malignant T4-2 cells showed increased signaling from EGFR and β1 integrin, relative to their nonmalignant predecessors, and that down-modulation of either EGFR or β1 integrin activity in cells grown in 3D lrBM caused the cells to form growth-arrested, polarized acinus-like structures (Weaver et al., 1997; Wang et al., 1998). As PI3K is an effector of both of these signaling pathways (Chen and Guan, 1994; Lee and Juliano, 2000; Grant et al., 2002), we hypothesized that inhibition of PI3K signaling would also revert these cells. We found that treatment of T4-2 cells with 8 μM of the PI3K inhibitor LY294002 (which prevented phosphorylation of downstream Akt at serine 473, as well as other downstream signaling mediators glycogen synthase kinase-3β [GSK-3β] and p70S6K; Fig. 1 B) did cause phenotypic reversion, as characterized by inhibition of proliferation, decreased colony size, and reduced growth in soft agar cultures (Fig. 1, A and C). Using indirect immunofluorescence, we found that the LY294002-reverted T4-2 cells regained the polarization of the apicolateral tight junction marker ZO-1, the basal marker α6 integrin, and the reorganization of the actin cytoskeleton (Fig. 2 A). Similar data were obtained in cells treated with the alternative PI3K inhibitor, wortmannin (unpublished data). These results demonstrate that down-regulation of the PI3K pathway in T4-2 mammary tumor cells restores an intrinsic property of forming polarized, growth-arrested structures in response to a physiologically relevant microenvironment.

Phenotypic reversion is accompanied by repolarization of PI3K and its phospholipid product

Proper interpretation of extracellular signaling cues requires asymmetric distribution of intracellular signaling molecules (Comer and Parent, 2002; Wedlich-Soldner and Li, 2003). Recently, signaling asymmetry of PI3K and its lipid product, PIP3 has been shown to control the directionality of chemotactic migration in human neutrophils and in single *Dictyostelium* cells (Servant et al., 2000; Funamoto et al., 2002; Wang et al., 2002b), and PIP3 has been found to become polarized to the basal surface of MDCK cells grown as monolayers on filters or in 3D collagen gels (Watton and Downward, 1999; Yu et al., 2003). We found that both PI3K (p85 subunit) and PIP3 are polarized to the basal surface of phenotypically normal S-1 cells grown in 3D lrBM, and that this asymmetric distribution is lost in T4-2 cells (Fig. 2 B). However, phenotypic reversion of the T4-2 cells through attenuation of PI3K signaling led to repolarization of these signaling components (Fig. 2 B). Given that the S-1 and reverted T4-2 cells show correct tissue polarity, whereas the untreated T4-2 cells are apolar, these results provide the

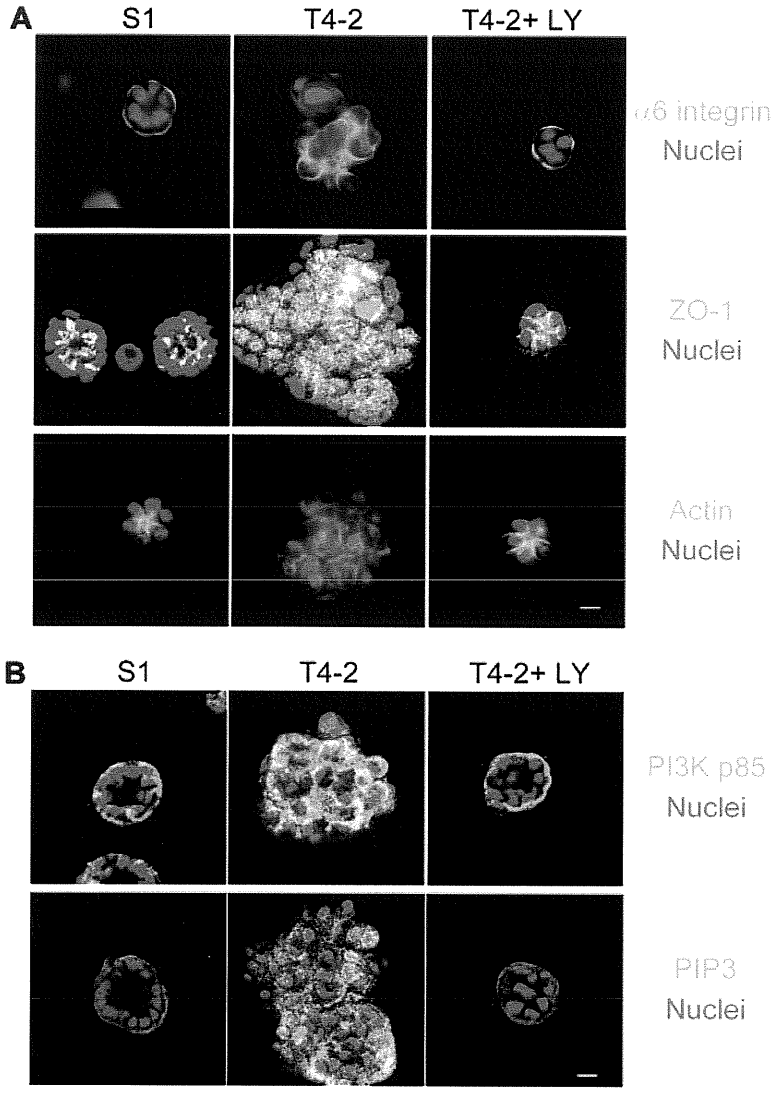

Figure 2. **Inhibition of PI3K results in reestablishment of tissue polarity.** (A) Down-modulation of PI3K activity of T4-2 cells was sufficient to repolarize the apicolateral tight junction protein ZO-1 and the basal ECM receptor, α6 integrin, and to result in the reorganization of the actin cytoskeleton. (B) PI3K and its phospholipid product, PIP3, are basolaterally localized in S-1 acini, apolarly distributed in the T4-2 colonies, and normalized in the reverted T4-2 structures. For both A and B, S-1, T4-2, and T4-2+ LY (revertants) were cultured for 10 d in 3D lrBM. α6 integrin, ZO-1, actin, PI3K p85 subunit, and PIP3 were stained by specific antibodies and phalloidin-FITC, and imaged by confocal fluorescence microscopy. Bars, 10 μm.

first evidence that polarized distribution of PI3K and PIP3 is an intrinsic property of phenotypically normal acini that is lost during tumor progression.

Phenotypic reversion of T4-2 cells by treatment with LY294002 results in cross-modulation of multiple signaling pathways

We had shown previously that inhibition of either EGFR or β1-integrin results in phenotypic reversion of T4-2 cells associated with down-modulation of the total levels of both signaling molecules, and that this activity influences and is influenced by the MAPK signaling pathway (Wang et al., 1998). We now show that PI3K signaling is also an integral component of this cross-modulated signaling network. T4-2 cells treated with LY294002 show reduced levels of EGFR and β1 integrin (Fig. 3 A). This effect depended upon 3D lrBM as it is not observed in cells cultured on two-dimensional (2D) plastic substrata (it should be noted that inhibition of PI3K activity, as measured by activation of downstream mediators Akt and GSK-3β, was equally effective in cells on 2D or in 3D; Fig. 3 A). In addition, our results revealed that PTEN, the antagonist of PI3K that acts to dephosphorylate PIP3 and which becomes down-regulated in many carcinomas (Simpson and Parsons, 2001; Yamada and Araki, 2001), is also a component of the cross-modulated signaling network, as treatment of T4-2 cells with LY294002 resulted in an increase of PTEN to the level of the nonmalignant cells; this modulation, too, was seen only in cells cultured on 3D lrBM (Fig. 3 B). Taken together, these results demonstrate the existence of a retrodirectional control network that exists only when cells are cultured in a proper tissue context.

Increased proliferation and loss of tissue polarity are functionally separable consequences of increased PI3K signaling

PI3K has been found to control a wide variety of downstream signal transduction pathways, the number and composition of which vary according to cell and tissue type (Chan et al., 1999; Vanhaesebroeck et al., 2001). The best-studied effector of PI3K is Akt, a regulator of cellular proliferation and apoptosis (Scheid and Woodgett, 2001). We examined the

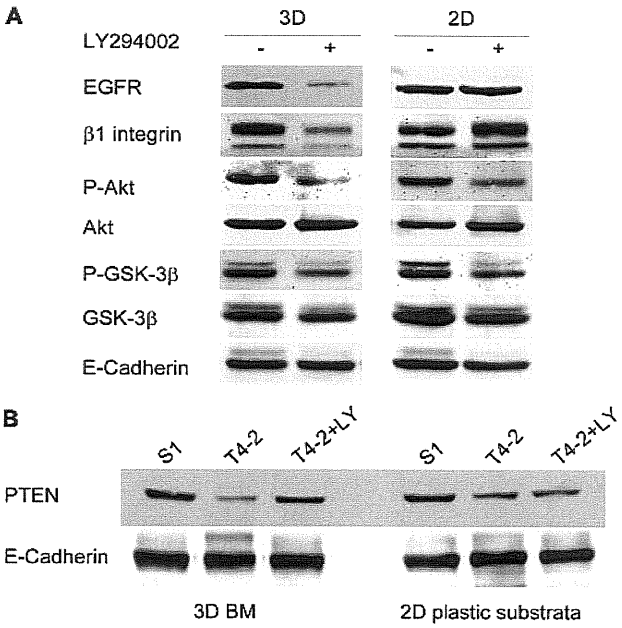

Figure 3. **Attenuation of PI3K activity results in cross-modulation of other signaling pathways and intermediates.** Cell lysates from S-1, T4-2, and T4-2+LY grown in 3D lrBM or on 2D plastic substrata for 10 d were analyzed for expression of (A) EGFR, β1 integrin, phosphorylated Akt (serine 473)/total, phosphorylated GSK-3β (serine 9)/total, and (B) PTEN ($n = 3$); E-cadherin was used as the loading control. It was shown previously that the total level of E-cadherin does not change under these conditions (Weaver et al., 1997).

possibility that the PI3K inhibitor-mediated reversion was due to reduction of Akt activity by expressing a dominant active Akt construct (Myr-Akt) in the T4-2 cells (Fig. 4 A). Expression of this construct blocked the effect of PI3K inhibitor on phosphorylation of Akt and downstream mediators of Akt activity (Fig. 4 B), and substantially increased the proliferation of T4-2 cells (Fig. 4, C and D). However, examination of colony polarity revealed that the LY294002-treated T4-Myr-Akt cell spheroids largely retained basal tissue polarity despite their considerably larger size (Fig. 4, E and F). This result revealed that increased proliferation alone was not sufficient to disrupt tissue polarity in the reverted T4-2 cells (which retain all of their phenotype-altering genetic mutations), and suggested that other effectors of PI3K might be responsible for disruption of tissue polarity.

Recent investigations of acinus-like cysts of MDCK cells grown in 3D collagen gels have demonstrated a role for Rac1 in control of cellular polarity (O'Brien et al., 2001). When we examined the activity of Rac1 in S1, T4-2, and LY294002-reverted T4-2 cells using pull-down assays, we found a high correlation of active Rac1 levels with loss of tissue polarity (Fig. 5 A). To test the role of Rac1 in the reversion phenotype, we expressed a dominant active Rac1 construct in T4-2 cells (Rac1L61; Fig. 5 B). We found that this construct did not greatly affect the rate of cellular proliferation (Fig. 5, C and D), but did inhibit the restoration of polarity in response to LY294002 (Fig. 5, E and F). Although the inhibition of reversion by the Rac1L61 was incomplete, this was most likely due to the heterogenous expression of this construct in the target cell population (assessed by immunofluorescence; unpublished data). As a complementary method to validate this result, we also found that infection with an adenovirus containing Rac1V12, which was expressed in a much higher proportion of the cells, showed much greater resistance to LY294002-mediated reversion of polarity (Fig. S1 C, available at http://www.jcb.org/cgi/content/full/jcb.200306090/DC1).

These results demonstrated that the increased PI3K signaling in T4-2 cells, relative to the same cells treated with LY294002 or to the nonmalignant S-1 cells, leads to activation of both Rac1 and Akt, and that these effectors signal to two functionally distinct phenotypes: activation of Rac1 causes the loss of cellular polarity, and activation of Akt causes increased proliferation. To determine whether together these two effectors were sufficient to recapitulate the effects of PI3K, T4-2 cells were infected with both constructs (Fig. 6 A). Colonies derived from cells transfected with these constructs were considerably larger than from control vector-infected cells. Treatment with PI3K inhibitor reduced the increased proliferation only to the level of the malignant vector-transfected cells (Fig. 6, B and C), but it had no repolarizing effect (Fig. 6 D). To determine whether these effects were also manifested in a different surrogate tumor malignancy assay, the four cell types (T4-2, T4+Myr-Akt, T4+Rac1L61, and T4+MyrAkt+Rac1L61) were cultured in 3D methylcellulose in the absence or presence of LY294002 (Fig. 6 E, −LY and +LY). In this assay as well, we found that combined expression of both constructs completely abrogated the effects of LY294002 treatment.

Discussion

A number of studies have shown that the PI3K signaling pathway becomes dysregulated in many types of carcinoma (Vivanco and Sawyers, 2002). The results presented here define mechanisms by which the high activity of PI3K in malignant T4-2 human breast cells contributes to their malignant phenotype. We find that down-modulation of PI3K activity in the T4-2 cells grown in 3D lrBM causes structural repolarization and reversion to a nonmalignant phenotype (Fig. 1) similar to the effects observed previously from inhibition of EGFR and β1 integrin (Weaver et al., 1997; Wang et al., 1998), and we additionally show that both the normal and the normalized reverted acinus-like structures had basal polarization of PI3K and its lipid signaling product, PIP3 (Fig. 2 B). The evidence that inhibition of PI3K can affect crossmodulation of a number of distinct signaling pathways is a demonstration that pathways downstream of PI3K are integrated into transduction networks when cells are grown in the physiological 3D lrBM; consistent with this model, we found that reversion of the tumor cells to a normal phenotype was associated with increased expression of PTEN, the PI3K antagonist (Fig. 3 B). Looking for the signaling effectors that controlled the increased proliferation and decreased polarity downstream of PI3K in the T4-2 cells led to identification of Akt as a mediator of increased proliferation (Fig. 4) and Rac1 as an inhibitor of polarization (Fig. 5), and we found that these two genes, when expressed in combination, were sufficient to overcome the inhibition of

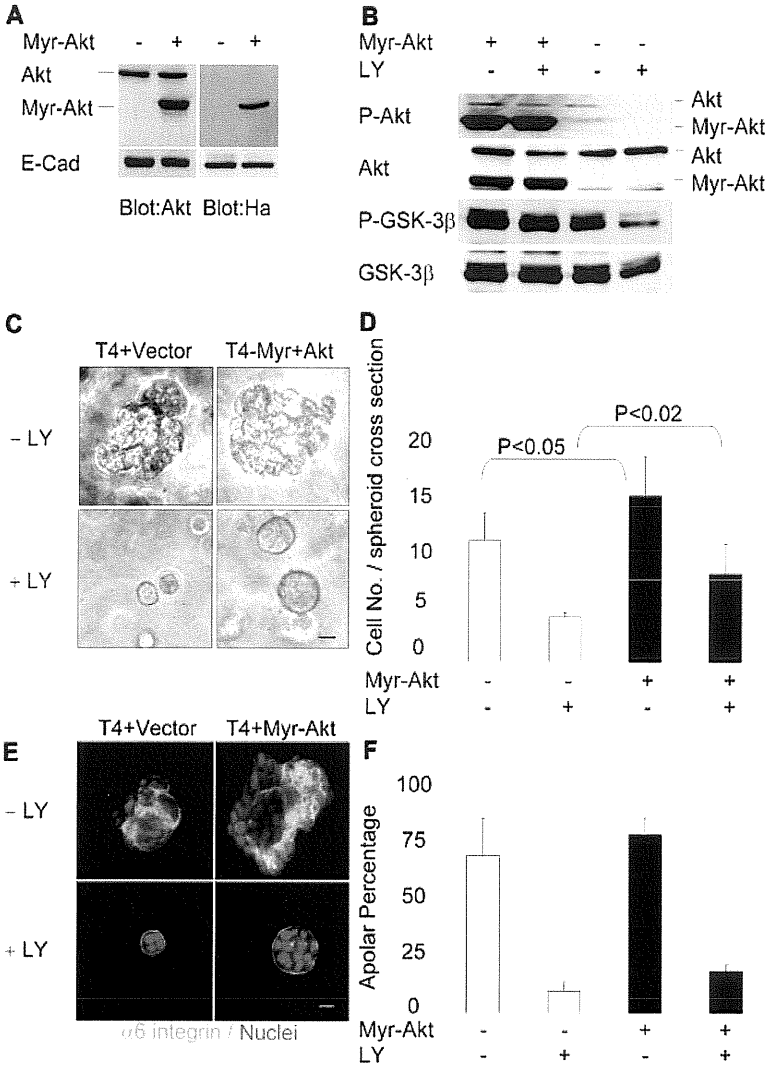

Figure 4. **Expression of Akt increases proliferation but does not affect polarity.** (A) Expression of constitutively active Akt in T4-2 cells (Myr-Akt), detected by Western analysis of cells infected with Myr-Akt (+) or vector control (−), and probed with anti-Akt (left) or anti-HA antibodies (right). (B) Activity of Myr-Akt mutant and its downstream target were not affected by PI3K inhibitor LY294002; cell lysates from T4-2 and T4-2+LY expressing Myr-Akt or vector grown in 3D lrBM for 10 d were analyzed for phosphorylated Akt (serine 473)/total and GSK-3β (serine 9)/total. (C) T4+Myr-Akt colonies were larger than control (T4-2+Vector) colonies, both in the presence and absence of LY294002, as assessed by phase contrast microscopy. Bar, 20 μm. (D) T4-2 + Myr-Akt colonies had more nuclei per spheroid cross section. Total nuclear number at spheroid cross section and spheroid numbers were counted and are presented as cell number per spheroid cross section. Statistical analyses revealed significant differences between Myr-Akt and vector control (mean ± SD, P values calculated using Student's *t* test; more than 500 colonies from 5 independent experiments were analyzed for each condition). (E) Constitutively active Akt signaling did not affect the basal tissue repolarization when T4-2+Myr-Akt cells were reverted by PI3K inhibitor, as assessed by basal localization of α6 integrin relative to DAPI-stained nuclei. Bar, 10 μm. (F) Quantitative analysis of polarity by percentage of spheroids without polarized distribution of basal α6 integrin. No significant difference was found between Myr-Akt and vector control for each condition (mean ± SD, $P > 0.05$, Student's *t* test; more than 600 colonies were analyzed for each condition from 3 independent experiments).

PI3K (Fig. 6). These results suggest a model in which the key aspects of the early malignant phenotype, growth and disorganization, can be controlled through disruption of signaling pathways that become interconnected and integrated in 3D lrBM (Fig. 7).

It is an important consideration, however, that the progressively increased proliferation and disorganization that typify acquisition of malignancy in mammary epithelial cells is due to more than just PI3K-dependent activation of Rac1 and Akt (although combined activation of these two pathways has been shown to be sufficient to confer malignancy in an experimental model of premalignant human mammary epithelial cells; Zhao et al., 2003). During tumor progression there are many genetic and epigenetic alterations that together contribute to produce malignancy; for the progression of S-1 to T4-2 cells, these include induction of many pathways likely not directly related to PI3K signaling. Reversion of the T4-2 tumor cells grown in 3D suppresses most (if not all) of these other pathways, reducing the proliferation and restoring polarity, but does not change the fact that the reverted cells still retain all of the same genetic alterations and abnormalities. Thus, reversion (whether by LY294002 or by any other T4-2 reversion reagent) results in a cell system that is genetically malignant but biochemically normal, and the consequences of manipulating a single signaling pathway in reverted cells provides information directly relevant to basic characteristics of malignant cell behavior. Using this model, we now show how the altered signaling pathways in T4-2 tumor cells may contribute to the tumor phenotype, how the functions of master regulators (such as PI3K) may be dissected in terms of downstream signaling pathways (in this case, Rac1 and Akt), and how manipulation of these pathways can cause the tumor cells to adopt a normal phenotype.

Normalization of signaling pathways in T4-2 cells in response to inhibition of PI3K is dependent upon culture in 3D lrBM, as T4-2 cells grown on 2D tissue culture plastic do not show the dramatic downmodulation of β1 integrin and EGFR (Fig. 3 A), up-regulation of PTEN (Fig. 3 B), or the alterations in cellular morphology in response to treatment with inhibitors of PI3K (Figs. 2 and 3). Also, for T4-2 cells grown in 3D lrBM, the reduction in PI3K signaling is paralleled by a reorganization of signaling orientation, as both PI3K and its phospholipid product, PIP3, became repolarized to the basolateral surface of the reorganized T4-2 cell

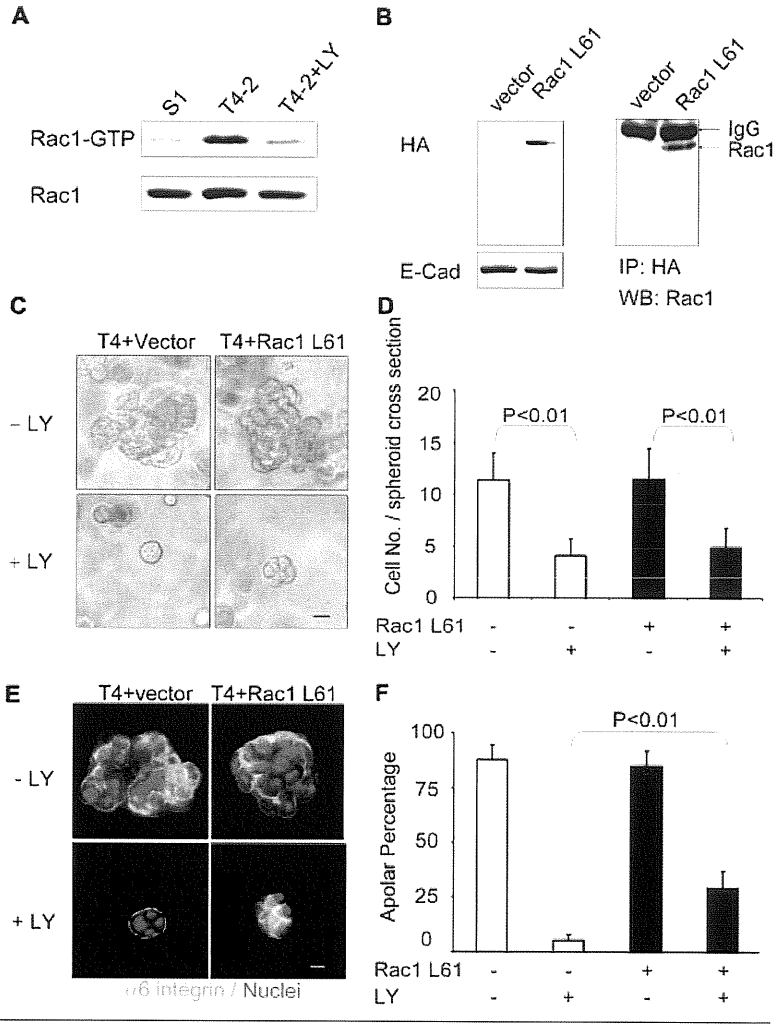

Figure 5. **Expression of Rac1 disrupts tissue polarity but does not prevent proliferation arrest.** (A) Rac1 activity correlates with PI3K activity, analyzed by using recombinant PAK-GST-CD fusion protein pulldown from lysates of 10-d 3D lrBM cultures of S-1, T4-2, and T4-2+LY cells. (B) Expression of constitutively active Rac1 L61, detected from lysates of cells infected with Rac1L61 or vector control and probed with anti-HA antibody (left) or immunoprecipitated with anti-HA antibody and blotted by anti-Rac1 antibody (right). (C) Inhibition of PI3K attenuates growth of both T4-2+Rac1 L61 colonies and control (T4-2+Vector) colonies, as assessed by phase contrast microscopy. Bar, 20 μm. (D) Analysis of total nuclear number at spheroid cross section reveals that treatment with LY294002 causes statistically signinficant decrease of colony cell number, but expression of Rac1L61 causes no substantial difference (mean ± SD, P values calculated using Student's *t* test; more than 300 colonies were analyzed from 3 independent experiments for each condition). (E) Attenuation of PI3K activity does not restore tissue polarity in T4-2 cells that express Rac1 L61, as assessed by immunofluorescence of vector control or transfected cells, stained with antibody against α6 integrin and with DAPI. Bar, 10 μm. (F) Statistical analysis of data in E, in which polarity was assessed by percentage of spheroids without polarized distribution of α6 integrin at basal surface (mean ± SD, $P < 0.01$, vector control versus Rac1 L61 cells treated with LY294002, Student's *t* test; more than 700 colonies were analyzed for each condition from 3 independent experiments).

structures (Fig. 2). This basolateral distribution of PI3K and PIP3 might indeed reflect the localization of active cell surface receptors, e.g., integrins and receptor tyrosine kinases, many of which have particular functions when localized to the basal or basolateral surfaces (Playford et al., 1996; Weaver et al., 1997; Vermeer et al., 2003). Basolateral polarization of PIP3 has been suggested to be a critical determinant of differentiated tissue behavior in polarized MDCK cells grown as monolayers on filters (Watton and Downward, 1999) or as cysts in 3D collagen gels (Yu et al., 2003), and PIP3 becomes apolarly distributed in the plasma membrane during branching morphogenesis (Yu et al., 2003), a process believed to involve the transitory dedifferentiation to a migratory and invasive state that is highly reminiscent of the malignant phenotype. PI3K signaling polarization is also an essential component of chemotactic migration in neutrophils and *Dictyostelium* (Servant et al., 2000; Funamoto et al., 2002; Wang et al., 2002b), and the directionality of neuronal axon growth is controlled by spatially localized PI3K activity (Shi et al., 2003). Our observations in mammary epithelial cells do not reveal the extent to which the polarized distribution of PI3K and PIP3 causes, or is the consequence of, tissue polarity, but previous observations with these cells in 3D lrBM and with MDCK cysts have suggested that formation of cell–cell contacts is an essential component of ECM-induced cell polarity (Weaver et al., 1997, 2002; Yeaman et al., 1999). If so, then formation of tight junctions at points of cell–cell contact may provide boundaries for localization of PI3K and other signaling effectors such as integrins and growth factors that then provide the polarizing principle. These possibilities are under investigation.

We have found that the 3D presentation of lrBM is essential for coupling the expression levels and activity of EGFR and β1 integrin in cultured mammary epithelial cells (Wang et al., 1998), and evidence in other systems also implicates reorganization of signaling pathways in cells cultured in 3D lrBM (Cukierman et al., 2001, 2002; Muthuswamy et al., 2001). We now show that components of the PI3K signaling pathway are involved in this cross-modulation process, as phenotypic reversion by inhibition of PI3K is associated with, and presumably, supported by, up-regulation of the PI3K antagonist, PTEN (Fig. 3). This also requires the establishment of organized structures in 3D lrBM, as treatment of T4-2 cells with PI3K inhibitors does not result in up-regulation of PTEN when cells are grown on 2D plastic substrata (Fig. 3 B). Given that the signaling reorganization associated with reversion of T4-2 cells is associated with global repolarization of signaling molecules, we suggest that di-

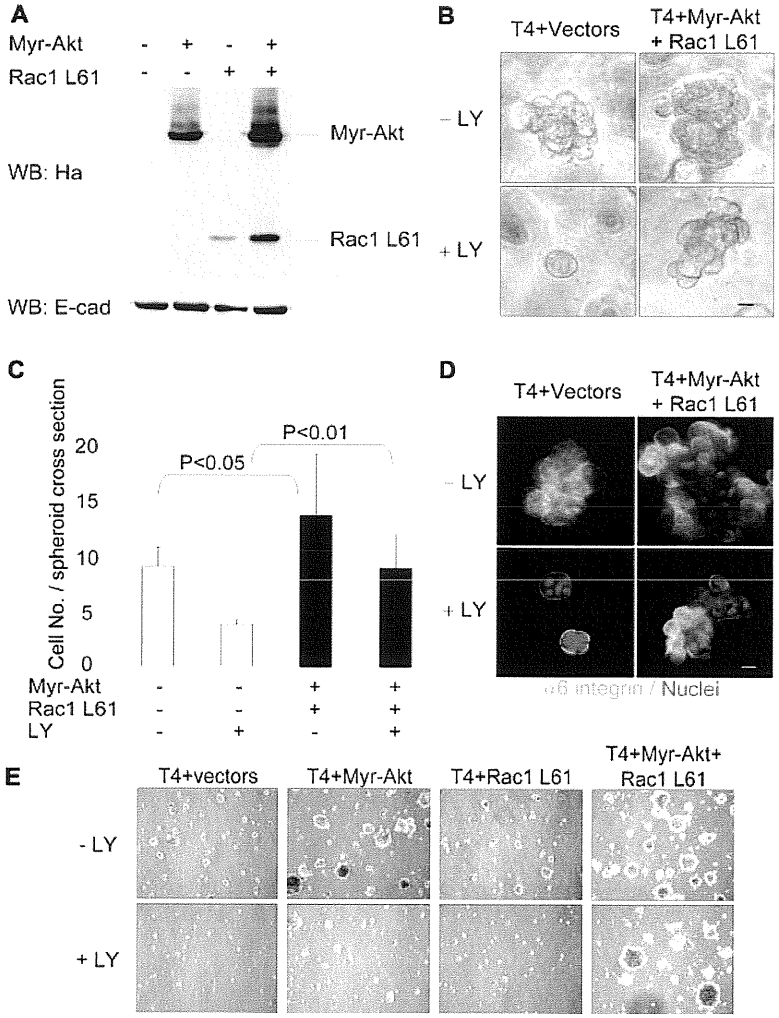

Figure 6. **Simultaneous expression of both Akt and Rac1 is sufficient to prevent reversion induced by attenuation of PI3K activity.** (A) Expression of both constitutively active Akt (Myr-Akt) and Rac1 (Rac1 L61) in T4-2 cells was detected by probing Western blots of cell lysates with anti-HA antibody. (B) PI3K inhibition fails to decrease proliferation and to restore tissue organization in Myr-Akt and Rac1 L61-double transfectants, as assessed by phase contrast microscopy and (C) by increased nuclei per spheroid cross section (mean ± SD, P values calculated using Student's *t* test; more than 300 colonies were analyzed for each condition from three independent experiments). Bar in B, 20 μm. (D) Restoration of basal tissue polarity is disrupted in double transfectants, as assessed by aberrant location of α6 integrin in LY294002-treated T4-2 Myr-Akt+Rac1 L61 cells grown in 3D lrBM for 10 d. Representative images from three independent experiments are shown. Bar, 10 μm. (E) Myr-Akt and Rac1 L61 double transfectants collaborate to increase anchorage-independent cell growth and overcome the inhibitory effects of treatment with PI3K inhibitor. Transfectants were grown in methyl cellulose for 3 wk in the absence or presence of PI3K inhibitor. Representative images are shown from duplicate experiments.

rectional orientation of signaling is an essential component of the cross-modulation process. In this regard, it is tempting to speculate that the 3D lrBM–directed basal localization of PI3K and PIP3 may explain why the cells in the outer layer of acini are more resistant to apoptosis than those not in contact with 3D lrBM (Debnath et al., 2002).

High expression of PI3K is commonly found in cancers and cancer cell lines (Vivanco and Sawyers, 2002; Wang et al., 2002a), and there is considerable evidence that the activity of this enzyme is a key component of the tumorigenic process. Cowden syndrome (an autosomal-dominant cancer predisposition syndrome caused by inherited mutations in PTEN) causes elevated risk of breast, thyroid, and skin tumors (Liaw et al., 1997); mice made heterozygous for expression of PTEN develop cancers at multiple sites (Di Cristofano et al., 1998), and transgenic mice deficient for PTEN expression in the mammary gland developed tumors at early stage (Li et al., 2002). Recent experiments using immortalized human mammary epithelial cells has shown that early passage cells require transfection of additional oncogenes along with PI3K (or Rac1/Akt) to become malignant, whereas late passage cells (which presumably accumulate more alterations) can be transformed with only PI3K (or Rac1/Akt) (Zhao et al., 2003); these results are also consistent with our model in which additional abnormalities must exist in addition to PI3K activation in order for HMT3522 mammary epithelial cells to become tumorigenic. Overexpression of constitutively active Akt in T lymphocytes, pancreatic cells, and the mammary gland increases cellular proliferation and promotes survival but does not induce cellular transformation or increase tumor incidence (Vivanco and Sawyers, 2002), suggesting that additional factors must be required. Rac1 isoforms are overexpressed in cancers of the breast and other organs, and increased activity of Rac1 or Rac3 has been found in breast carcinoma cell lines and Ras-transformed breast epithelial cells (Mira et al., 2000; Sahai and Marshall, 2002). Here, we have unified the roles of elevated Rac1 and Akt activities in a simple mechanistic framework: we have found that the high levels of PI3K in T4-2 cells contribute to the loss of polarity and increased proliferation through Akt and Rac1, and we find that these pathways and phenomena are functionally separable. We find that PI3K-Akt signaling is responsible for an appreciable increase in cell proliferation (Fig. 4), whereas the PI3K-Rac1 signaling is responsible for the loss of basal tissue polarity (Fig. 5), and that expression of both can completely prevent reversion by LY294002. These results show that overactive PI3K signaling activates these two effectors for separate but

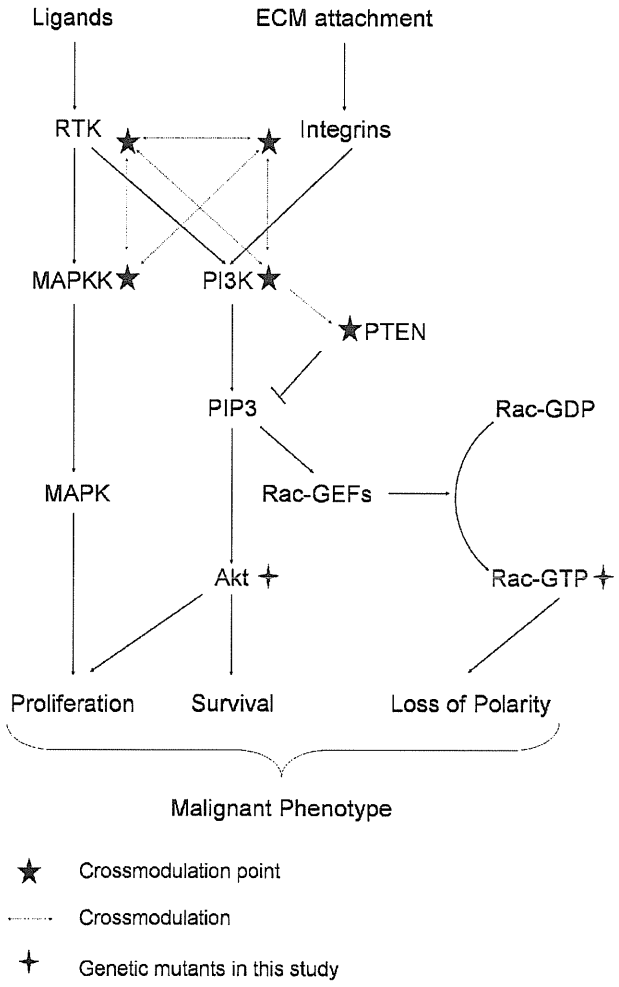

Figure 7. **Scheme of proposed tumor cell signaling network to control polarity, proliferation, and apoptosis.** T4-2 signaling network. PI3K activity is increased in tumor cells as a result of aberrant signaling from cell–ECM and cell–growth factor receptor interactions. The consequent activation of Akt contributes to the increased cellular proliferation through downstream pro-proliferation and anti-apoptotic pathways. Increased PIP3 also leads to up-regulation of Rac1 through activation of Rac1-specific GEF activity, resulting in altered organization of the actin cytoskeleton, formation and maintenance of tight junctions, and directionality of vesicle trafficking, effects that combine to disorganize the tissue structure. Thus, each pathway independently affects cellular behavior, but the synergistic effect leads to the tumor phenotype. (★) Cross-modulation in 3D lrBM. Inhibition at any of these pressure points results in normalization of the expression and activity at each of the other points, with concomitant normalization of downstream signaling pathways.

collaborative regulation of the distinct cellular behaviors of tumor tissues (Fig. 7). Significantly, our results also reveal that increased cell proliferation (in the absence of a polarity-disrupting signal) is not sufficient to result in loss of tissue organization (Fig. 4 E), a finding that may explain why Akt overexpression by itself is not sufficient to increase tumor incidence as well.

In conclusion, we have used the 3D lrBM assay to determine the role of PI3K signaling in the tumorigenic phenotype, signaling reorganization, and tissue polarity of mammary epithelial cells. Our discovery of asymmetric distribution of PI3K and PIP3 in these polarized acinus-like structures strongly implies that they might act as spatial determinants to regulate mammary epithelial polarity. Our elucidation of the events downstream of PI3K sheds light on the process by which increased proliferation and loss of tissue polarity act collaboratively to produce the malignant phenotype.

Materials and methods

Reagents and cell culture

lrBM from Englebreth-Holm-Swarm tumors (matrigel), Vitrogen (rat tail collagen type I), and the antibodies for E-cadherin, β1-integrin, EGFR, α6 integrin, and Ki-67 were described previously (Weaver et al., 1997; Wang et al., 1998). The other antibodies used in this study are: PTEN (clone 2), Akt (clone 7), Rac1 (clone 102), PI3K p85 (clone 4), and p110 subunit (clone 19) (Transduction Laboratory), HA (12CA5; Roche), Phospho-Akt (serine 473 or threonine 308), total and phospho-GSK-3β (serine 9), total and phospho-p70 S6 kinase (threonine 389; Cell Signaling Technology), PIP3 monoclonal antibody (Chen et al., 2002; clone RC6F8; Echelon Corp.), and ZO-1 antibody (gift of Shoichiro Tsukita, Kyoto University Faculty of Medicine, Kyoto, Japan). HMT-3522 mammary epithelial cells were cultured as described previously (Weaver et al., 1997; Wang et al., 1998). The PI3K inhibitor LY294002 or wortmannin (Calbiochem) was dissolved in DMSO and added to culture medium at the final concentration of 8 μM or 2.5 nM after cells were plated; control cultures were treated with vehicle only. Cells were treated with inhibitor or vehicle inside matrigel or on tissue culture plates for 10 d and inhibition was maintained by replacing with fresh medium containing inhibitor or vehicle every 2 d. No toxicity was found during the 10-d treatment with LY294002 or wortmannin.

DNA constructs and gene transfection

A construct containing Rac1 L61–HA (gift of Tung C. Chan, Thomas Jefferson University, Philadephia, PA) was digested with EcoRI and BamHI and cloned into the pLXSN–Neo retroviral construct (CLONTECH Laboratories, Inc.). The myr-Akt pWZL retroviral construct myrΔ4–129 (Kohn et al., 1998) was provided by Richard Roth (Stanford University, Stanford, CA). Transfection of Phoenix packaging cells (gift of Garry P. Nolan, Stanford University) and production of retroviral stock were according to standard protocols. The HMT-3522 mammary epithelial cells were infected at 40–50% confluence. Myr-Akt and Rac1 L61-double transfectants were produced by sequentially infecting cells with each construct. The stably expressing cells were selected in the presence of neomycin (500 μg/ml) or hygromycin B (50 μg/ml) and surviving clones were pooled.

Immunoblotting, immunoprecipitation, and indirect immunofluorescence

Immunoblotting and indirect immunofluorescence were performed as described previously (Weaver et al., 1997; Wang et al., 1998). For immunoprecipitation, cells were lysed in IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E 64, and 1 mM Pefabloc) and centrifuged at 16,000 *g* at 4°C. Equal amounts of protein lysates were precleared by 50 μl of protein G plus–conjugated agarose beads (Santa Cruz Biotechnology, Inc.) before the addition of 1 μg of primary antibody. Samples were incubated at 4°C with gentle rotation for 1 h. Subsequently, samples were incubated with 30 μl of protein G plus–conjugated beads for 1 h at 4°C. The beads were washed three times with IP buffer before being heated with sample buffer at 95°C for 5 min and analyzed by SDS-PAGE and Western blotting.

For Rac1 activity assay, the cells from 10-d culture in 3D BM were lysed in GST-Fish buffer (10% glycerol, 50 mM Tris, pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E 64, and 1 mM Pefabloc) and centrifuged at 16,000 *g* at 4°C. Equal amounts of protein supernatants were incubated with recombinant GST-PAK-CD fusion protein (containing the Rac and Cdc42 binding region from human PAK1; Sander et al., 1998), bound to glutathione-coupled Sepharose beads (Amersham Biosciences) at 4°C for 30 min. The beads were washed with an excess of lysis buffer, eluted in sample buffer, and then analyzed by SDS-PAGE and Western blotting using antibody against Rac1.

For PIP3 immunofluorescence, the isolated colonies were fixed with 3.7% formaldehyde, washed with CSK buffer (10 mM Hepes, 138 mM

KCl, 3 mM $MgCl_2$, 1 mM EDTA), permeabilized by 0.1% Triton X-100 in CSK buffer, and blocked with 3% skim milk in blocking buffer (50 mM Tris/HCl, pH 7.5, 1 mM $CaCl_2$). Primary antibody diluted 1:100 in blocking buffer was incubated with samples for 1 h in room temperature followed by FITC-conjugated secondary antibody.

Nuclei were counterstained with DAPI (Sigma-Aldrich). Control sections were stained with secondary antibodies only. The slides were sealed with Vectashield (Vector Laboratories). The images were collected with Zeiss 410 LSM confocal microscope (Zeiss Pluar 40× oil objective lenses; Carl Zeiss MicroImaging, Inc.) or RT SLIDER SPOT digital camera (SPOT RT v3.2 software; Diagnostic Instruments) attached to Zeiss Photomicroscope III (Zeiss Plan-Neofluar 40× oil objective lenses; Carl Zeiss MicroImaging, Inc.). Images for figures were colored and resized with Adobe Photoshop 7.0 software.

Anchorage-independent growth assays

For soft agar assay, 5,000 cells were plated in 1 ml of DME/F12 containing 0.3% agarose, overlaid with 1 ml of 1% agarose, and then exposed to treatment as indicated. Cultures were maintained for 15 d. Colonies from duplicated wells were measured and scored positive when the colony sizes exceeded a diameter of 50 μm.

For methyl cellulose anchorage–independent growth assay, 100,000 cells were seeded per 60-mm dishes in 5 ml of DME/F12 containing 1.5% methyl cellulose (Fisher Scientific) with inhibitor or vehicle only. Colonies were scored after 3 wk.

Online supplemental material

Supplemental figures show the following results; treatment of T4-2 cells with PI3K inhibitor LY294002 at 8-μM concentration when grown in 3D lrBM for 10 d does not affect cell viability or lead to increased cell death (Fig. S1 A); the same treatment decreases cell proliferation of T4-2 cells when cultured on 2D plastic (Fig. S1 B); expression of constitutively active Rac1 V12 in T4-2 cells transduced by adenovirus inhibits LY294002-induced repolarization of basal marker α6 integrin in 3D lrBM (Fig. S1 C); treatment of T4-2 cells with PI3K inhibitor does not change its expression levels (Fig. S2 A); overexpression of constitutively active Akt in T4-2 cells greatly increases anchorage-independent growth of T4-2 cells in soft agar (Fig. S2 B); and PIP3 staining intensity is significantly reduced when treating T4-2 cells with PI3K inhibitor (Fig. S2 C). Figs. S1 and S2 are available at http://www.jcb.org/cgi/content/full/jcb.200306090/DC1.

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Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2

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Akt1 is frequently up-regulated in human tumors and has been shown to accelerate cell proliferation and to suppress programmed cell death; consequently, inhibition of the activity of Akt1 has been seen as an attractive target for therapeutic intervention. Paradoxically, hyperactivation of the Akt1 oncogene can also prevent the invasive behavior that underlies progression to metastasis. Here we show that overexpression of activated myr-Akt1 in human breast cancer cells phosphorylates and thereby targets the tumor suppressor tuberous sclerosis complex 2 (TSC2) for degradation, leading to reduced Rho-GTPase activity, decreased actin stress fibers and focal adhesions, and reduced motility and invasion. Overexpression of TSC2 rescues the migration phenotype of myr-Akt1-expressing tumor cells, and high levels of TSC2 in breast cancer patients correlate with increased metastasis and reduced survival. These data indicate that the functional properties of genes designated as oncogenes or tumor suppressor genes depend on the context of the cell type and the tissues studied, and suggest the need for caution in designing therapies targeting the function of individual genes in epithelial tissues.

metastasis | oncogene | tumor suppressor

Metastasis occurs when cancer cells invade beyond the boundaries of the primary site and establish new tumors in distant organs; because metastases are responsible for most cancer deaths, attention has focused on the mechanisms by which cancer cells acquire invasive metastatic properties (1). Invasion by cancer cells into surrounding tissues involves alterations in cellular shape and stiffness that facilitate interaction with the new tissue microenvironment (2), and these modifications depend on reorganization of the actin cytoskeleton through the action of Rho-GTPases (3). Although 22 members of the Rho-GTPase family have been identified, it is the activation of the Rho subfamily that is principally responsible for the assembly of the contractile actomyosin machinery necessary for most types of cell motility (4). Inhibition of Rho or its effector Rho-kinase (ROCK) significantly compromises cellular migration and invasiveness (5–10), and increased expression/activation of Rho and its isoforms has been found in many types of cancers (11–14). A recently discovered mechanism for activating Rho in epithelial cells involves the tuberous sclerosis complex 2 gene product, TSC2 (15).

Tuberous sclerosis complex is a disorder characterized by hamartomas in the skin, kidney, heart, and lung and is caused by inactivating mutations in the tumor suppressor genes TSC1 and TSC2 (16). Although it has been best characterized as a controller of cell size through its regulation of ribosomal synthesis proteins including ribosomal S6 kinase (17, 18), TSC2 has been implicated in a number of related signaling pathways in a variety of cell types (19). Investigations of Madin–Darby canine kidney epithelial cells demonstrated that overexpression of TSC2 results in activation of Rho and increased cellular motility (15), and analysis of TSC2-deficient cells has shown reduced Rho activity (16), although these effects may depend on the cell type investigated (20). Although mutational inactivation of TSC2 contributes to tuberous sclerosis complex, regulation of intact TSC2 occurs principally through the action of the serine/threonine kinase Akt1/protein kinase B (21, 22). Phosphorylation of TSC2 by Akt1 stimulates association with 14-3-3 proteins that mediate subcellular translocation and proteolytic degradation of TSC2 (23–27).

Akt1 is one of the most frequently activated protein kinases in human cancer (28, 29). Activation of Akt1 is associated with resistance to apoptosis, as well as increased cell growth, proliferation, and energy metabolism (28, 30). Activated Akt1 is correlated with altered cell migration and invasion in several mammalian systems: constitutively active Akt1 or its isoforms can enhance the ability to invade in already invasive cultured cancer cells (31–33), but Akt1 can have the opposite effect on normal or less invasive cells (33). Moreover, a constitutively active allele of Akt1 blocks ErbB2/Neu-mediated invasion into surrounding tissue and lung metastasis in double transgenic mice (34). These results reinforce the idea that understanding cell- and tissue-specific signaling pathways is critical for evaluating the implications of activated upstream signaling molecules on complex phenotypic effects (35).

In a study investigating the mechanisms involved in the development of functional mammary epithelial cell structures, we found that signaling pathways activated by phosphatidyl inositol 3-kinase (PI3K) disrupted normal tissue structure by blocking polarity through increased Rac1 activity and stimulating cell proliferation through increased Akt1 activity (36). We found that cells expressing activated Akt1 showed the expected effects of increased proliferation and resistance to apoptosis but surprisingly also appeared to have substantially decreased invasiveness and motility. Here we show that inhibition of motility by activated Akt1 depends on down-regulation of Rho activity. We further show that TSC2 is a key intermediate in this mechanism, that activated Akt1 inhibits motility by stimulating the degradation of TSC2, and that increased expression of TSC2 can block the invasion-inhibitory effects of activated Akt1.

Results

Activated Akt1 Promotes Cell Proliferation, Survival, and Tumor Growth but Inhibits Cell Motility and Invasion. Akt1 has been implicated in multiple cellular processes, including cell survival, proliferation, growth, and apoptosis resistance (28, 37). The particular role it plays in a given tissue, however, depends on the model system investigated. We expressed constitutively activated, myristoylated Akt1 (myr-Akt1) (38) or empty vector in the human mammary epithelial cancer cell line HMT-3522 T4-2 (T4-2) (39, 40). In these pooled populations, increased Akt1

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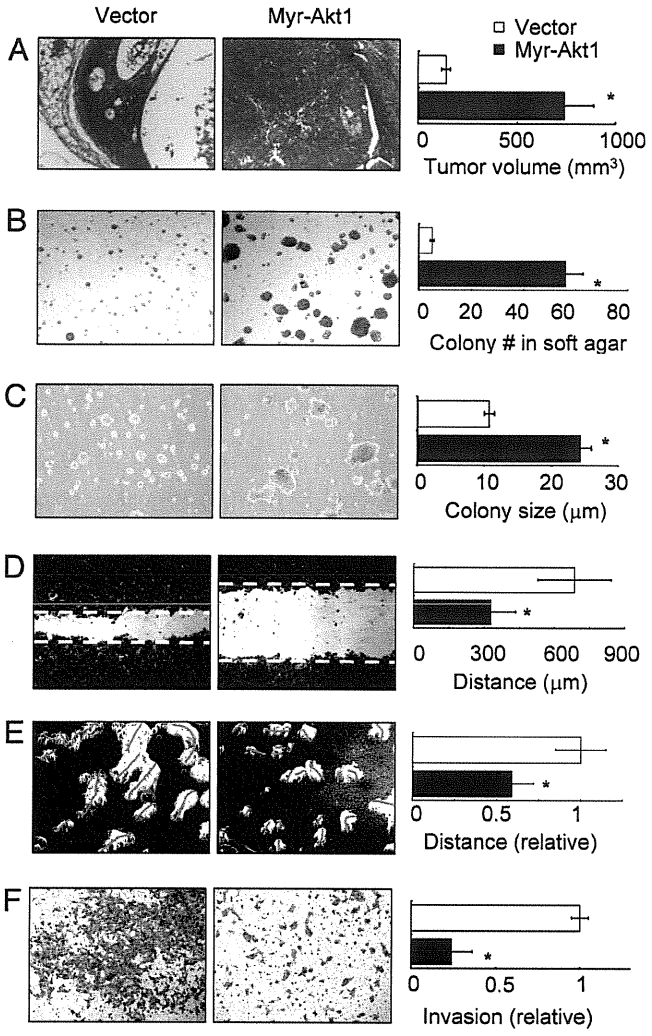

Fig. 1. Activated Akt1 promotes growth and anchorage independence in T4-2 cells but suppresses motility and invasion. Cells expressing myr-Akt1 showed increased tumor volume in nude mouse xenografts (*A*), increased colony number in methyl cellulose (*B*), and increased colony size in soft agar (*C*). However, myr-Akt1 inhibited cell migration (*D*) and motility (*E*), as well as invasion (*F*). All graphs display averages ± SEM. *, $P < 0.05$.

signaling induced by this construct was confirmed by phosphorylation of Akt1 substrates and specific downstream targets (Fig. 6, which is published as supporting information on the PNAS web site). Consistent with the results seen with the transgenic Akt1 mice (34), xenografts of myr-Akt1-expressing cells formed tumors that develop more rapidly and to a much larger size than control cells ($P < 0.001$; Fig. 1*A* and data not shown). Cells overexpressing myr-Akt1 showed significantly increased colony number and size in methylcellulose and soft agar assays (Fig. 1 *B* and *C*). When cultured in 3D laminin-rich extracellular matrix (lrECM) (Matrigel or collagen I+laminin1), cells expressing myr-Akt1 also showed larger colony size (36) (data not shown) because of increased cell proliferation and suppressed apoptosis as assessed by KI-67 and TUNEL immunostaining (Fig. 7, which is published as supporting information on the PNAS web site). These results demonstrate that Akt1 activation strongly stimulates cell survival and tumor growth in this experimental model. Opposite effects were seen in cell migration, motility, and invasion assays, however. Myr-Akt1 significantly inhibited cell motility in wound-healing assays (Fig. 1*D*), and by tracking cell

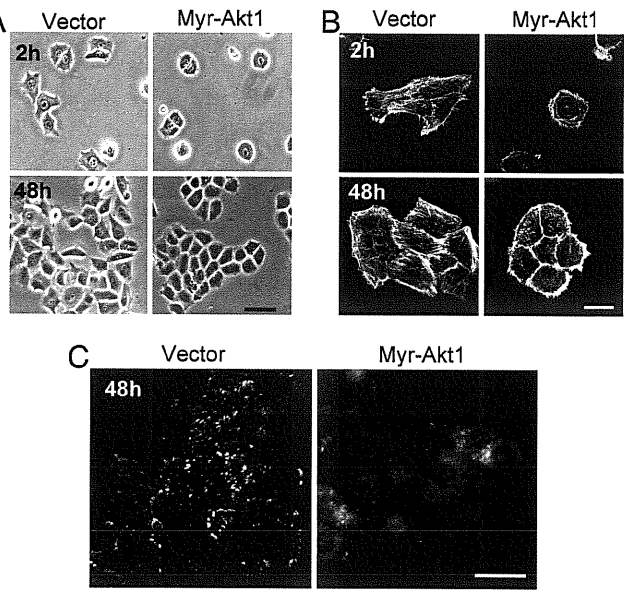

Fig. 2. Activated Akt1 alters cell-substratum adhesion. Cells expressing myr-Akt1 show reduced cell spreading (*A*), increased cortical distribution of actin (*B*), and fewer paxillin-containing focal adhesions (*C*). (Scale bars: *A* and *C*, 10 μm; *B*, 20 μm.)

location for 24 h, we found that the motility of isolated myr-Akt1 cells was significantly reduced relative to control cells (Fig. 1*E*), which suggests that the decreased migration in the scratch assay is not due to increased cell–cell adhesion. Consistent with these observations, we found that active Akt1 significantly reduced invasion of T4-2 cells through Matrigel-coated Boyden chambers (Fig. 1*F*). Previous studies have linked Akt activity to expression of matrix metalloproteinases (MMPs) (31), well known effectors of cell invasiveness, and although we found that cells expressing myr-Akt1 showed reduced expression of MMP-9, supplementation with much higher levels of MMP-9 in the media did not rescue the inhibition of motility by myr-Akt1 (data not shown).

Cell Spreading and Formation of Focal Adhesions Are Inhibited by Activated Akt. Cell motility is a dynamic process in which moving cells undergo dramatic changes in cell shape, associated with the creation of actin-rich protrusions, formation and disassembly of adhesive complexes, and establishment of migration polarity (2, 41). We found that cell spreading was greatly reduced in cells expressing myr-Akt1 (Fig. 2*A*); because actin polymerization is one of the major forces involved in cell spreading (42), we evaluated the actin cytoskeleton in myr-Akt1-expressing cells. We found that cells expressing active Akt1 had increased cortical actin staining and reduced stress fiber formation (Fig. 2*B*), and that initiation of cell movement in scratch assays was associated with fewer actin-rich projections toward the scratch wound in myr-Akt1 cells (data not shown). Reduced cell extensions in myr-Akt1 cells in the cell spreading and motility assays are consistent with the requirement for these structures during the onset and maintenance of cell motility (2).

Focal adhesions are required for cell attachment and serve as points of contact for cell spreading and traction during migration (43); we examined the effect on focal adhesion formation in cells expressing myr-Akt1 by immunostaining for paxillin. We determined that activated Akt1 decreased the formation of focal adhesions without affecting the level of endogenous paxillin expression (Fig. 2*C* and data not shown). These results suggest that reduced motility in myr-Akt1 cells is due to alterations in the assembly and function of the actin cytoskeleton.

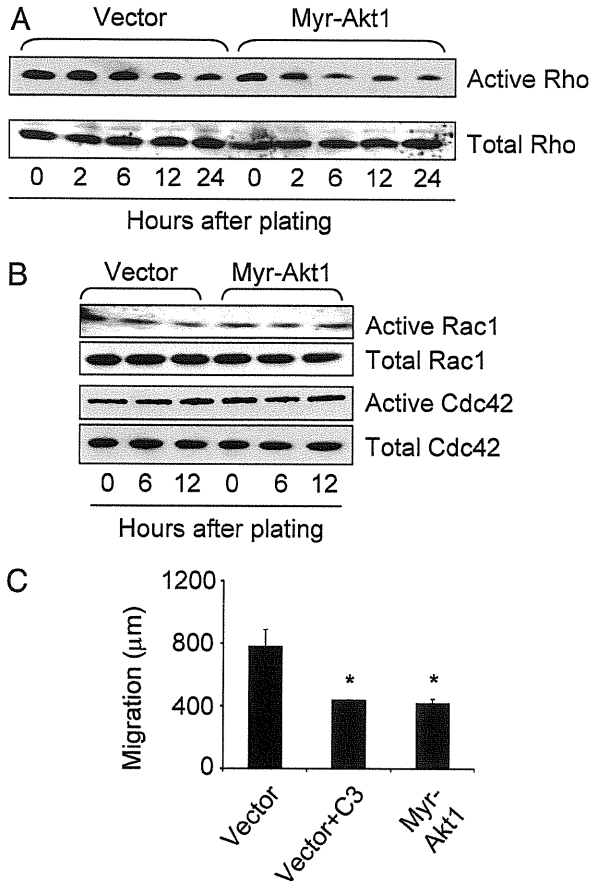

Fig. 3. Rho GTPases are selectively inhibited by Akt1. Cells were plated for the indicated times, then lysates were analyzed for activity by pull-down with GST-Rhotekin (*A*) or GST-PAK-CD (*B*). (*C*) Inhibition of Rho by pretreatment with recombinant C3 transferase (10 μg/ml) reduced migration of vector control cells in scratch assay to levels of myr-Akt1-expressing cells. The graph displays the average ± SEM. *, $P < 0.05$.

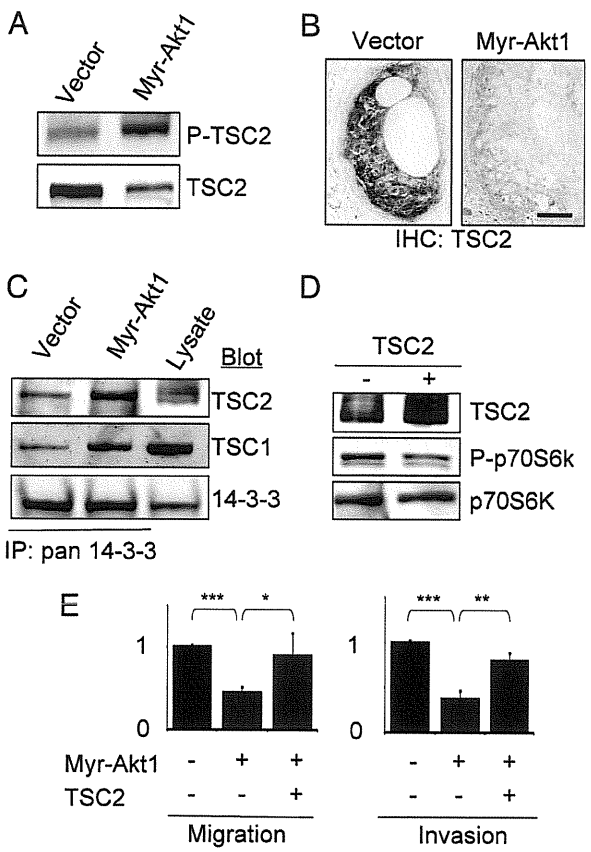

Fig. 4. TSC2 mediates the effects of Akt1 on motility and invasion. (*A*) TSC2 shows increased phosphorylation but decreased total levels in cells expressing myr-Akt1. (*B*) Decreased levels of TSC2 in tumor xenografts from myr-Akt1 cells. (Scale bar: 50 μm.) (*C*) TSC2 shows increased association with 14-3-3 in cells expressing myr-Akt1. (*D*) Expression of functional TSC2 in myr-Akt1 cells as shown by reduced phosphorylation of p70S6K. (*E*) Expression of TSC2 rescues cell invasiveness and motility in myr-Akt1 cells. The graphs display averages ± SEM. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.001$.

Active Akt1 Inhibits Rho. Assembly and organization of the actin cytoskeleton are controlled by Rho GTPases, and the formation of stress fibers and focal adhesions is a characteristic effect of activation of Rho GTPases (44). Using a Rhotekin-GST fusion protein pull-down assay, we found that active Rho levels were significantly lower in myr-Akt1-expressing cells, starting 2 h after cell plating (Fig. 3*A*). To determine the effect of the decreased Rho activity on actin organization, cells plated on coverslips were stained with phalloidin at the same time points as pull-down assays. At all time points, myr-Akt1-expressing cells spread more slowly and formed fewer stress fibers than vector control cells (Fig. 2 *A* and *B* and data not shown). We found no substantial difference in Rac1 or Cdc42 activities (Fig. 3*B*). Consistent with the implication that compromised cell motility and invasiveness in myr-Akt1-expressing cells was due to inhibition of Rho activity, treatment with exoenzyme C3 transferase (C3 toxin), which inhibits Rho when added to the media (45, 46), was as effective at blocking cell migration as was expression of myr-Akt1 (Fig. 3*C*).

Active Akt1 Controls Migration and Invasion by Modulating Levels of the Tumor Suppressor TSC2. In many systems, Akt1 has been shown to phosphorylate TSC2, triggering 14-3-3-dependent proteolytic degradation (16, 19). Moreover, elevated TSC2 and TSC1 have been shown to increase Rho activity in epithelial Madin–Darby canine kidney cells (15) and in fibroblasts (47), respectively. Accordingly, we hypothesized that Akt1-mediated inhibition of Rho activity was the consequence of phosphorylation and down-modulation of TSC2. Consistent with this possibility, we found increased phosphorylation and decreased total levels of TSC2 in cells expressing myr-Akt1 (Fig. 4*A*), and decreased TSC2 expression in myr-Akt1 tumor xenografts (Fig. 4*B*). Using immunoprecipitation experiments, we found significantly increased association of TSC2 with 14-3-3 in cells expressing myr-Akt1 (Fig. 4*C*).

To determine whether exogenous expression of TSC2 could compensate for the inhibition of cell motility and invasion induced by active Akt1, wild-type TSC2 was stably expressed in vector control and myr-Akt1 cells (Fig. 4*E*). Functional expression of TSC2 was demonstrated by decreased phosphorylation of p70S6K (Fig. 4*D*) (48). We found that myr-Akt1 cells expressing TSC2 showed enhanced migration and invasion (Fig. 4*E*), as well as increased cell spreading, formation of stress fibers, and focal adhesions (Fig. 8, which is published as supporting information on the PNAS web site).

Relative Expression of Akt1 and TSC2 Predicts Progression to Metastasis in Human Breast Cancer Patients. These results suggested a model (Fig. 5*A*) in which active Akt1 reduces the levels of TSC2, which causes a reduction in the TSC2-dependent activation of Rho, causing in turn decreased cell migration, motility, and

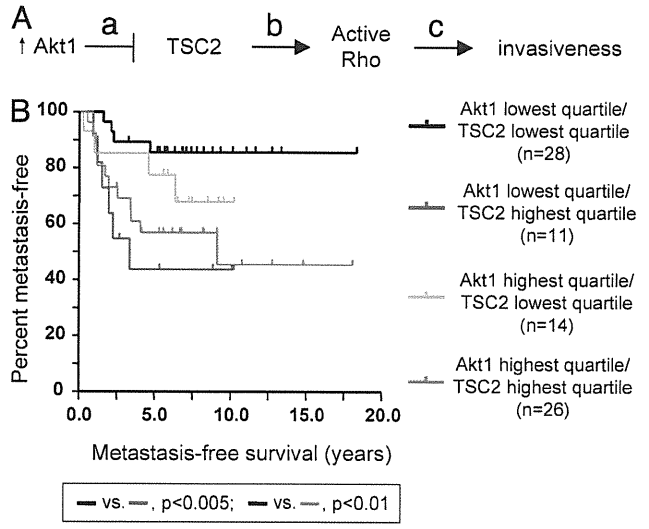

Fig. 5. Analysis of pathway implication on human breast cancer metastasis. (*A*) Pathway model. a, Active Akt1 phosphorylates TSC2, stimulating down-modulation of TSC2 levels; b, TSC2 activates Rho; c, Active Rho promotes cytoskeletal rearrangements permissive for cell invasion. (*B*) Metastasis-free survival of human breast tumors stratified by expression of Akt1 and TSC2. Higher expression of TSC2 together with low expression of Akt1 is predictive of decreased time to metastasis in breast tumors.

invasiveness. This system presents a striking example of the importance of cell-type specificity for the function of key signaling molecules, inasmuch as the expression of the oncogene Akt1 is inhibiting the invasive behavior that underlies progression to metastasis, whereas the tumor suppressor TSC2 is stimulating this same tumor progression step. Moreover, because the inhibition of Rho activity and consequent effects on invasiveness depend on the relative activities of Akt1 and TSC2, this model suggests that the effect of TSC2 to stimulate the pro-metastatic invasive phenotype would be most pronounced in cells expressing lower levels of Akt1.

To evaluate the applicability of these findings, we interrogated a comprehensive RNA expression microarray data set of 295 primary human breast cancers (49). To analyze the contribution of TSC2 and Akt1 to tumor progression and metastasis, tumors were divided in quartiles by expression level of each marker, and metastasis-free survival was calculated for the upper and lower quartiles ($n = 74$ samples for each single gene). Although elevated expression of TSC2 did appear to correlate with decreased time to metastasis (data not shown), this result fell short of the standard for statistical significance ($P = 0.053$). However, consideration of Akt1 expression levels in combination with TSC2 expression produced a different result (Fig. 5*B*). We found that tumors in both the highest quartile for TSC2 expression and the lowest quartile for Akt1 (blue line, $n = 11$) showed significantly decreased time to metastasis relative to tumors in the lowest quartile of TSC2 and Akt1 expression (black line, $n = 28$; $P < 0.005$). Comparison of tumors with high TSC2 and high Akt1 (red line, $n = 26$) to tumors with low TSC2 and high Akt1 (green line, $n = 14$), by contrast, revealed no statistically significant difference.

Discussion

Akt1 has been classified as an oncogene on the basis of its frequent up-regulation in a wide variety of human tumors (reviewed in refs. 50 and 51), but increased expression of Akt1 does not always correlate with invasiveness (52, 53), the defining stage of malignancy. Recent experimental studies have confirmed these observations: comparisons of ErbB2/Akt1 double transgenic mice with ErbB2 single transgenics have shown that expression of activated Akt1 accelerates tumorigenicity but blocks metastasis (34), and in articles appearing after this work was completed, expression of activated Akt1 was shown to inhibit motility and invasiveness of MDA-MB-435 (54) and MCF-10A (55) cells. These observations are corroborated by other studies of cultured cancer cells, because almost without exception, increased activation of Akt1 correlates with increased proliferation and anchorage-independent growth, but the effects of activated Akt1 on cell migration and invasiveness depend on the cell type that is investigated (31–33, 54–57). Here, we present a mechanism to account for this apparent paradox: overexpression of an activated isoform of Akt1 leads to phosphorylation and degradation of TSC2 and decreased Rho activity, resulting in a reduction in stress fiber and focal adhesion formation, reduced random and persistent migration, and invasion (Fig. 5*A*).

Such observations suggest the existence of a dichotomy between proliferation and cell motility. Although often regulated by the same extracellular cues and intracellular signal transduction pathways, cell migration and proliferation rarely occur simultaneously. During wound healing, keratinocytes migrate into the wound site before proliferating (58), and during embryonic development, neural crest cells can migrate across the distance of the embryo without entering the cell cycle (59). Additional examples can be found during development of the lung and vasculature, where buds and sprouts have been seen to form before initiation of cell proliferation (60, 61). Similarly, a switch between proliferation and migration has been observed in gliomas, other brain tumors, and bladder carcinoma (62–64). There are several possible explanations for this phenomenon. Much of the cytoskeletal machinery required for migration is also required for cell-cycle progression and cytokinesis. In addition to regulating the actin cytoskeleton during locomotion, RhoA accumulates at, and recruits myosin to, the cleavage furrow during cell division (65). It is conceivable that maintaining the cell in a nonmotile state during cell-cycle progression allows for the proper distribution of RhoA and other resources to facilitate cytokinesis, and this organization may constitute part of the checkpoint machinery. Our data implicate Akt1/TSC2 as a crucial signaling node in this decision-making process; a similar role has been reported for the adaptor protein Shc (66). If so, a breakdown of the separation of proliferative and invasive behaviors, as is observed in advanced stages of tumor progression, could contribute to improper apportioning of chromosomes during cell division, leading to genomic amplifications and deletions, thereby accelerating cancer progression.

Much of the current research into cancer etiology, progression, and potential therapies is centered on understanding and circumventing the activation of protooncogenes and for reactivating or substituting for the loss of protective tumor suppressor genes. However, classification of a gene as an oncogene or tumor suppressor may result from limited observations of specific cell lines, transgenic mouse tissues, or types of human tumors. Given that cancer is not a single disease, evaluating the status of a tumor simply by the presence of certain activated "oncogenes" or inactivated "tumor suppressors" is incomplete and potentially misleading. Indeed, a similar effect has been proposed for KLF4, which acts as a tumor suppressor in gastrointestinal cancer but has oncogenic properties in breast cancer cells (67); similarly, cell context-dependent tumor-suppressive and oncogenic effects have been found for TGF-β (68) and oncostatin (69). These findings suggest the importance of a more nuanced approach, because the functional outcome of activating or inactivating a gene depends largely on the cell type, its preexisting genetic background, and the local microenvironment (70). Here, the overexpression of Akt1 (a classical oncogene) leads to larger tumors that are less invasive, and that (given that the majority of mortality resulting from breast cancer is a result of metastatic

disease) could potentially lead to a more favorable outcome clinically if occurring in the absence of other tumor-specific modifications (34). Conversely, increased expression of TSC2 (a classical tumor suppressor) stimulates invasiveness and correlates with a less favorable clinical outcome (Fig. 5*B*). Ongoing efforts to therapeutically target the Akt1 pathway in breast cancer patients (71) might benefit from consideration of TSC2 expression status in the target population, inasmuch as reducing Akt1 activity in tumors expressing high levels of TSC2 could potentially enhance progression to a more malignant phenotype.

Materials and Methods

Cell Line, Constructs, and Antibodies. HMT-3522 mammary epithelial cells were cultured as described in refs. 40 and 72. The myr-Akt1 pWZL retroviral construct myrΔ4–129 and kinase-dead Akt1 (T308A/S473A) constructs were kindly provided by Richard Roth (Stanford University). TSC2/pMSCVneo was a gift from Elizabeth P. Henske (Fox Chase Cancer Center, Philadelphia). Transfection of Phoenix packaging cells (gift from Garry P. Nolan, Stanford University), production of retroviral stock, and viral infection were according to standard protocol. The stably expressing cells were selected in the presence of neomycin (500 μg/ml) or hygromycin B (50 μg/ml), and surviving clones were pooled. Antibodies were E-cadherin, paxillin (clone 165), hamartin (BD Biosciences), total and phospho-p70 S6 kinase (threonine 389), phospho-(Ser/Thr) Akt1 substrate, total and phospho-FKHR (serine 256), phospho-tuberin (threonine 1462) (Cell Signaling Technology), Akt1 and tuberin (C-20; Santa Cruz Biotechnology), 14-3-3 pan Ab-4 (clone CG15; NeoMarkers). Rho antibody was clone 55 from BD Biosciences, which does not distinguish between RhoA, RhoB, and RhoC.

Immunoblot Analysis and Immunoprecipitation. Immunoblot analysis was performed described in ref. 72. For immunoprecipitation, cells were lysed in IP buffer [1% Triton X-100/150 mM NaCl/10 mM Tris·HCl (pH 7.4)/1 mM EDTA/1 mM EGTA, containing 2 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E 64, and 1 mM Pefabloc]. Equal amounts of protein lysates were precleared by 50 μl of Protein G Plus-conjugated agarose beads (Santa Cruz Biotechnology) before the addition of primary antibody. Samples were incubated at 4°C for 1 h. Subsequently, samples were incubated with Protein G Plus-conjugated beads for 1 h at 4°C. The beads were washed three times with IP buffer before being heated with sample buffer and analyzed by SDS/PAGE and Western blotting.

Immunofluorescence and Immunohistochemistry. Cells were fixed either in 2% paraformaldehyde at room temperature for 20 min or in 1:1 methanol/acetone at −20°C for 10 min. The samples were blocked with blocking buffer (130 mM NaCl/15 mM Na_2HPO_4/3.5 mM NaH_2PO_4/0.1% BSA/0.2% Triton X-100/0.05% Tween 20) containing 10% goat serum and then incubated with primary antibodies followed directly by either FITC- or Texas red-conjugated secondary antibodies for 45 min. Nuclei were counterstained with 4′,6-diamidino-2 phenylindole (DAPI) (Sigma). The images were collected with a Zeiss 410 LSM confocal microscope (Zeiss Pluar ×40 oil objective lenses) or a SPOT RT Slider digital camera (SPOT V.3.2 software; Diagnostic Instruments, Sterling Heights, MI) attached to a Zeiss Photomicroscope III (Zeiss Plan Neofluar ×40 oil objective lenses).

Invasion and Migration Assays. For invasion assay, cell-culture inserts (8 μm, 24-well format; BD Discovery Labware) were evenly coated with diluted Matrigel. Cells (1×10^5) were added to the upper chamber, and the lower chamber was filled with 300 μl of medium. The culture was maintained for 48 h. The number of cells was counted under ×20 objective in each of five randomly chosen fields. Alternatively, the membrane of the insert was cut, and the dye was eluted from the membrane with 200 μl of 10% acetic acid. The OD reading was obtained at a wavelength of 590 nm. The stained insert without adding cells was used as background reading. Each experiment was duplicated. The cell migration assay is similar to the invasion assay except that inserts were not coated with Matrigel and that 10% FBS was added to lower chamber and the culture was maintained for 24 h. For cell-migration wound-healing assays, cells were plated into 24-well plates (Nalge Nunc) at the density of 4×10^4 cells per cm^2. After cells had grown to confluence, a wound was made across the well with blue tips under the guide of a ruler. The gap of the wound was marked and imaged immediately after wounding and 24 h later. The gap was measured and presented as net closure per 24 h. Each experiment was performed in duplicate or triplicate.

Anchorage-Independence Growth Assays. For soft agar assay, 5,000 cells were plated in 1 ml of DMEM/F12 containing 0.3% agarose, overlaid with 1 ml of 1% agarose. Cultures were maintained for 15 days. Colonies from duplicated wells were measured and scored positive when the colony sizes exceeded a diameter of 50 μm. For methyl cellulose assay, 100,000 cells were seeded per 60-mm dish in 5 ml of DMEM/F12 containing 1.5% methyl cellulose (Fisher Scientific). Colonies were scored after 2 weeks.

Xenografts. Nude mice, 4–5 weeks of age, were purchased from Simonsen Laboratories (Santa Clara, CA). T4-2 cells expressing myr-Akt1 or vector alone were propagated as monolayers, trypsinized, and resuspended in PBS at the concentration of 1×10^8 cells per ml. Then 0.1 ml of cell suspension (1×10^7 cells) was injected s.c. into rear flanks of nude mice, and tumor size was monitored twice per week by measuring tumor size (length × width × height). All of the mice were killed by CO_2 at day 17 because of tumor burden. Tumors were dissected, fixed in 2% formaldehyde, paraffin-embedded, and sectioned (5 μm).

GST Fusion Protein Pull-Down Assay. The cells were lysed in GST-Fish buffer [10% glycerol/50 mM Tris (pH 7.4)/100 mM NaCl/1% Nonidet P-40/2 mM $MgCl_2$, containing 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E 64, and 1 mM Pefabloc) and centrifuged at 16,000 × *g* at 4°C. Equal amounts of protein supernatants were incubated with recombinant GST-Rhotekin or GST-Pak-CD fusion protein-coupled Sepharose beads at 4°C for 45 min. The beads were washed with an excess of lysis buffer, eluted in sample buffer, and analyzed by SDS/PAGE and Western blotting with antibody against Rho, Rac1, or Cdc42.

Statistics. All data analysis was performed by using PRISM (GraphPad, San Diego). Bar graphs represent means ± SEM. The database consisting of the microarray profiles of the 295 human breast tumors and associated clinical data (49) was obtained from Rosetta Inpharmatics. Patients were stratified into quartiles for expression of TSC2 or Akt1, and metastasis-free survival was computed by using the Kaplan–Meier method; statistical significance was assessed by using the log-rank test.

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